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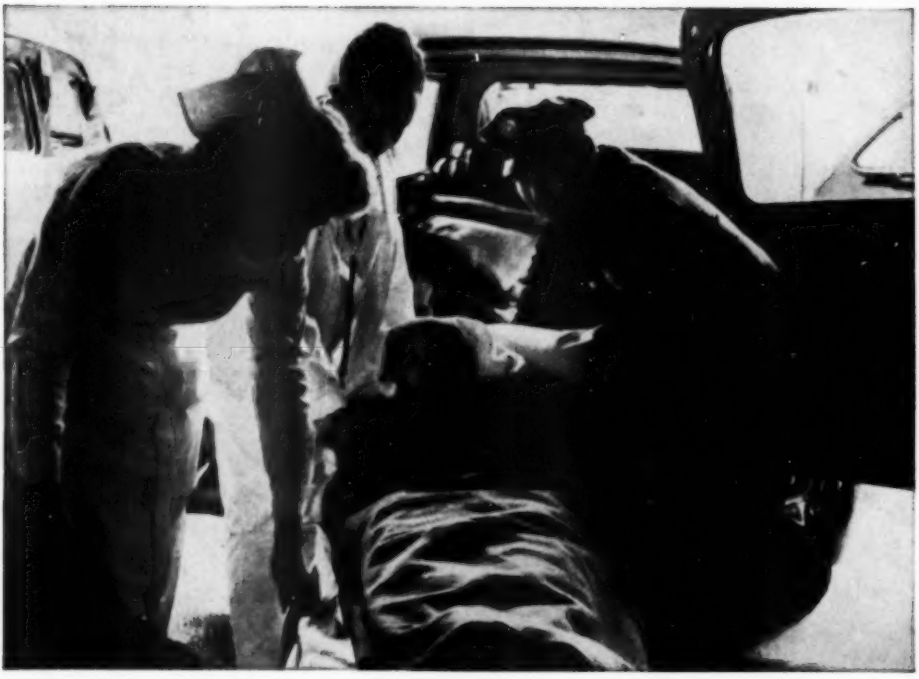

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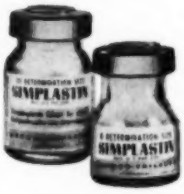

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MECHANISM OF SOFTENING OF TUBERCLES

III. Hydrolysis of Protein and Nucleic Acid During Anaerobic Autolysis of Normal and Tuberculous Lung Tissue in Vitro

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AND
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PHILADELPHIA

PRIOR to our investigations (Weiss and Boyar-Manstein,[1] Weiss and Singer [2]), three attempts were made to study the chemical processes concerned in the delayed autolysis (softening) of caseous tubercles. The first was that of Schmoll [3] (1904). He removed tuberculous caseous lymph nodes from cattle and emulsified the material with water to form a thin *brei*. After 26 days' autolysis only small increases were observed in total and basic nitrogen. Schmoll concluded that autolysis of caseous material is very sluggish, since, "it is dead tissue in the midst of the living organism." Since Schmoll's tissue extracts were very dilute, it is not surprising that they showed virtually no enzymatic activity and only minute amounts of soluble protein. He concluded that caseous tuberculous material consists almost entirely of coagulated protein with a high percentage of lecithin (3.83%) and no nucleoproteins or histones.

Noteworthy progress was made (1908) by Opie and Barker.[4] They produced tuberculosis in dogs by intrapleural injection of virulent bacilli, collected tuberculous tissue from the mediastinum and used it as the source of enzyme. The substrate was 50% heated blood serum. The authors concluded that the epithelioid cells of the tubercle contain an enzyme, lymphoprotease, which is active in acid reaction. It is present during the early stages of tuberculous infection but disappears as the process of caseation advances.

> It is not improbable that caseation which, like autolysis, is accompanied by disappearance of nuclei, is in part dependent on the presence in the cells of this active proteolytic enzyme which is for a time held in check. Injury to cells by products of the tubercle bacillus (or partial anaemia, the result of imperfect vascularization of the tuberculous tissue), may have a part in rendering these cells susceptible to self-digestion. Changes which have been observed in serum of the tuberculous exudate show that the anti-enzymotic property of the normal blood may be absent in the exudate of a tuberculous lesion. This loss of anti-enzymotic action, perhaps referable to changes caused by products of the tubercle bacillus, may favor self-digestion of the enzyme-containing cells and diffusion of their enzyme.

From the Laboratories of Microbiology, Albert Einstein Medical Center, Northern Division.

Presented before the American Association of Immunologists, Chicago, April 8, 1953; abstracted Fed. Proc. **12**:465, 1953.

This study was made possible by grants from the Committee on Medical Research and Therapy of the American Trudeau Society, Medical Section of the National Tuberculosis Association, and from the National Institutes of Health, United States Public Health Service.

Several years later, (1922) Opie * commented on his own work as follows: "There are no observations to show that enzymes such as leucoprotease which are inhibited by the anti-enzyme of the blood serum are concerned in the absorption of the tubercle." Rich [7] (1951) pointed out that it has not been proved that enzymes disappear from caseous material rapidly enough to cause failure of autolysis, nor has the presence of inhibitors been adequately ruled out.

Jobling and Petersen [8] (1914) employed human tuberculous lymph nodes and tissue removed at autopsy from areas of caseous pneumonia for their studies on autolysis. Unfortunately, they dried and pulverized their material at temperatures near 45 C., which may have inactivated many of the proteolytic and nucleolytic enzymes. They failed to obtain evidence of autolysis in dilute emulsions of caseous matter or of lymph nodes which had not become secondarily infected and which had been adjusted to either an "acid" or "alkaline" reaction. (The pH was not determined.) Tissue from areas of caseous pneumonia showed autolysis in "acid" but not in "alkaline" reaction. Jobling and Petersen suggested that soaps of unsaturated fatty acids function as inhibitors of proteolytic enzymes,[9] a theory later disproved by Teale and Bach,[10] cited by Opie,† and others.

In previous investigations Weiss and Boyar-Manstein [1] and Weiss and Singer [2] characterized certain proteolytic and nucleolytic enzymes of normal and tuberculous lung tissue and studied their quantitative distribution in the constituent parts of discrete tubercles. They demonstrated greatly increased enzymatic activity (benzoyl-*l*-arginine amidase [BA-amidase], leucine-amino-peptidase [LA-peptidase], and desoxyribonuclease [DNase]) in the inflammatory zones of the tubercles. In the centers of the tubercles where there was gross evidence of caseation, the rate of substrate cleavage was markedly decreased. The values for the rate of hydrolysis of LA-peptidase were very low and those for BA-amidase and DNase reached zero in areas where macroscopic softening had developed. In the course of this work, a DNase inhibitor was found in an aqueous extract of tubercle bacilli. Similarly, caseous and softened material removed from tubercles was found to contain inhibitors for BA-amidase and DNase.

In the above studies we investigated those enzymes most likely to function as catalysts in the hydrolysis of proteins and nucleic acids during softening. We used as substrates synthetic peptides and purified animal deoxyribonucleic acid (DNA). In the present report, the native proteins and nucleic acids in tissue homogenates were permitted to undergo autolysis in vitro and the pH optimum for these processes was found to be about 5.0. The in situ pH of various areas of discrete tubercles was determined and our previous findings regarding the distribution of the proteolytic and nucleolytic enzymes in tuberculous lung were confirmed and extended.

MATERIALS AND METHODS

Infection of Animals and Preparation of Tissue Homogenates.—As in our previous studies, young female albino rabbits were exposed to a virulent Ravenel strain of Myobacterium tuberculosis in the Wells apparatus for the transmission of air-borne, quantitative infection. With this method, one can obtain a small number (approximately 10) of discrete tubercles. Only with this type of lesion can one perform the work recorded below.

* References 5 and 6.

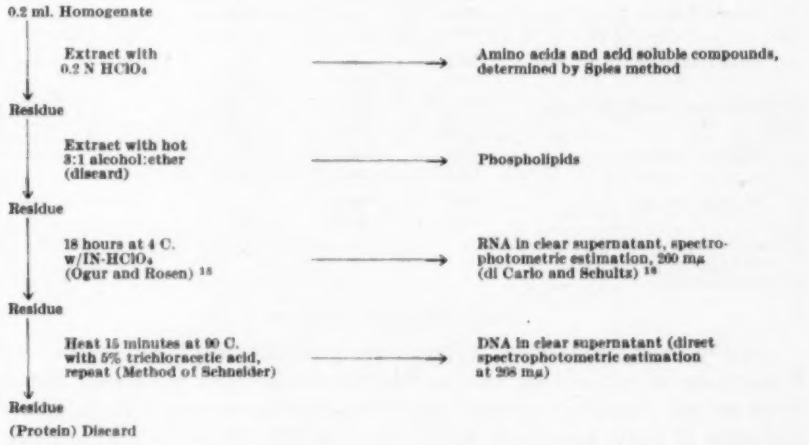
† References 5 and 6.

The tissues were stored at —30 C. until needed. Immediately preceding analysis, the individual caseous tubercles were excised and the surrounding inflammatory zones removed and examined separately. It was not possible to remove all traces of inflammatory tissue without great loss of caseous material. This may account for a portion of the activity of the latter.

In order to avoid an error of earlier workers, we used the minimal amount of buffer solution which permitted manipulation of the homogenates. In this way dilution of the enzymes and loss of activity were kept at a minimum. All tissues were kept ice-cold during homogenation.

One gram of lung tissue was homogenized in a Potter glass homogenizer with 2 ml. of the appropriate buffer (citrate, 0.06 M; acetate, 0.2 M; borate, a mixture of 0.05 M $Na_2B_4O_7.10H_2O$ and 0.2 M H_2BO_3) to give the desired pH and 1 cm. layer of toluene was poured over the homogenate contained in 10 × 100 mm. cotton plugged sterile test tubes. The tubes were placed in an anaerobic jar; the air was evacuated and replaced with hydrogen gas.

Autolysis was studied under anaerobic conditions because (*a*) intracellular animal proteinases (cathepsins) are more active under these conditions, (*b*) there is less likelihood of the development of enzyme-inhibitors (Irving, Fruton and Bergmann,[11] 1941), and (*c*) in the centers of the relatively avascular, caseous tubercles there is presumed to be a condition of lower oxygen tension (Loebel and co-workers, 1933).[12] The tubes were kept at 35 C. Periodically, aliquots of 0.2 ml. were transferred to 15 ml. centrifuge tubes in order to follow the rate of autolysis. The aliquots were fractionated as follows:



Method for the Study of Protein Hydrolysis.—The first fraction removed from the 0.2 ml. aliquot contained the amino acids released during proteolysis. These acids were removed by three extractions with 0.2 N $HClO_4$ (3 ml. plus 3 ml. plus 2 ml.) and the resulting extract neutralized with 1 N NaOH and made up to 10 ml. The concentration of α-amino acids in the extract was determined at 230 mμ by the copper method of Spies[13] and by the Grassmann-Heyde[14] titration. Although purines and pyrimidines interfered with the Spies method, the amounts present in test samples were too low to cause serious difficulty. Protein nitrogen was determined by a micro-Kjeldahl method.

Method for the Study of Nucleic Acid Hydrolysis.—After removal of the phospholipid fraction with alcohol and ether (3:1), 5 ml of 1 N $HClO_4$ was added to the residue and the tubes stored 18 hours at 4 C. (Ogur and Rosen,[15] 1950). This extract, containing ribonucleic acid (RNA), was cleared by centrifugation and the residue washed once with an additional 5 ml. of 1 N $HClO_4$. RNA was determined at 260 mμ by the method of di Carlo and Schultz,[16] (1948). RNA from the Nutritional Biochemical Corporation was used as standard. The residue from the RNA extraction was heated to 90 C. for 15 min. with 5 ml. of 5% trichloroacetic acid (TCA) and centrifuged. The heating was repeated, giving 10 ml. of a clear deoxyribonucleic acid (DNA) extract (Schneider,[17] 1946). The concentration of DNA present was usually too low

to be determined accurately by means of the Dische diphenylamine reagent. The method found best was to determine the DNA directly in the Beckman spectrophotometer at 268 mμ, using as standard pure DNA from the Worthington Biochemical Co. Heated TCA was used as a blank.

As a check on the above procedures, RNA and DNA phosphorus was determined in these extracts by a modification of the method of King [18] (1932). Since 5 ml. aliquots of the nucleic acid extracts were needed to give sufficient phosphorus for the tests, it was found necessary to neutralize partially the 1 N $HClO_4$ extract after hydrolysis of the organic phosphorus in order to develop the full blue color with molybdate. Ferrous sulfate (Sumner,[19] 1944) was used as reducing agent.

The values RNA and DNA obtained from various homogenates prior to incubation at 35 C. are given in Table 1. The direct spectrophotometric method gave slightly higher results for the nucleic acids, if we assume the phosphorus content of RNA and DNA to be between 9 and 10%. An average of over 60 determinations gave a value of 8% phosphorus for both RNA and DNA when results similar to those shown in Table 1 were calculated.

TABLE 1.—*Nucleoprotein Content of Normal and Tuberculous Lung Tissue*

Tissue	Compound	γ Nucleic Acid*	γ P*	% P‡	Ratio RNA:DNA
Normal lung	RNA	82.5	8.9	10.8	0.51
	DNA	161.0	15.0	9.3	
Inflammatory zone	RNA	138.0	10.0	7.3	0.74
	DNA	186.0	16.0	8.6	
Uninvolved tuberculous lung	RNA	123.0	7.7	6.3	0.57
	DNA	215.0	19.0	8.9	
Caseous material	RNA	95.0	6.5	6.8	0.49
	DNA	195.0	13.0	6.7	
Softened material	RNA	105.0	5.4	5.1	0.34
	DNA	305.0	25.6	8.4	

* In 0.1 ml. of homogenate.
‡ In nucleic acid.

EXPERIMENTAL RESULTS

A. Protein Hydrolysis.—In Figure 1 are presented the pH activity curves for homogenates of normal lung, caseous material, and contents of softened tubercles. After six days' incubation, the optimum for anaerobic proteolysis by the intracellular cathepsins of rabbit lung tissue was at about pH 5.0. From the graphs it can be estimated that at pH's between 6.6 and 7.1, which is the in vivo pH range for these tissues (see data and discussion below), there is approximately only 25% of maximal enzymatic activity.

Having established the optimal pH for proteolysis in tuberculous lung, we followed the progress of autolysis of material removed from caseous and softened tubercles. As shown in Figure 2, the inflammatory zone of tubercles has an activity far exceeding that of the other areas of a tuberculous lung, probably because this area contains a large concentration of intact phagocytic cells. Caseous tissue has approximately one-third the activity of inflammatory zone tissue. Some of this activity may be due to small bits of tissue containing intact cells which could not be removed from caseous tubercles without serious loss of material. Although the pH was optimal, there was very little proteolytic activity in normal lung or in the contents of softened tubercles. This may be due to a difference in the type of protein substrate available, since all of the homogenates contained approximately equal amounts of protein nitrogen per milliliter (about 0.5 mg. per 0.1 ml. of homogenate).

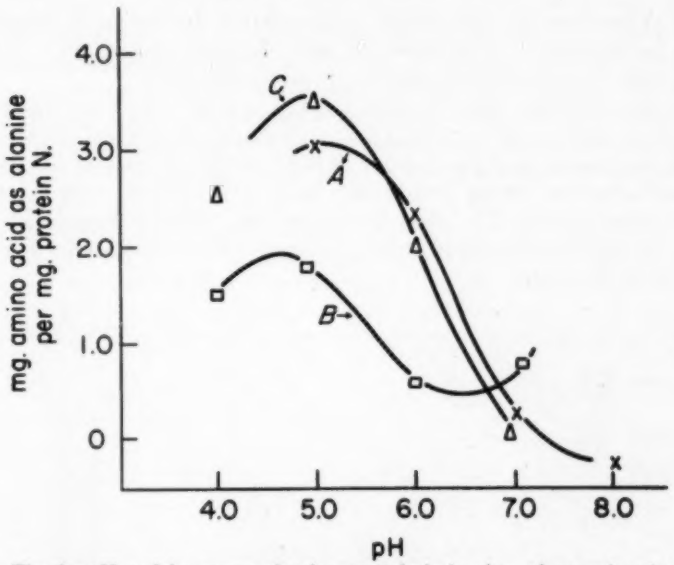


Fig. 1.—pH activity curves showing proteolysis in vitro of normal and tuberculous lung tissue. Ordinates indicate amino-acids released from native tissue proteins during six days' autolysis under anaerobic conditions. Curve *A*, caseous material; *B*, contents of softened tubercles; *C*, normal lung tissue. Data for *C* (in this figure only) are based upon values from Grassmann-Heyde titrations. Ordinates indicate ml. 0.01 N -NaOH per milligram of protein N.

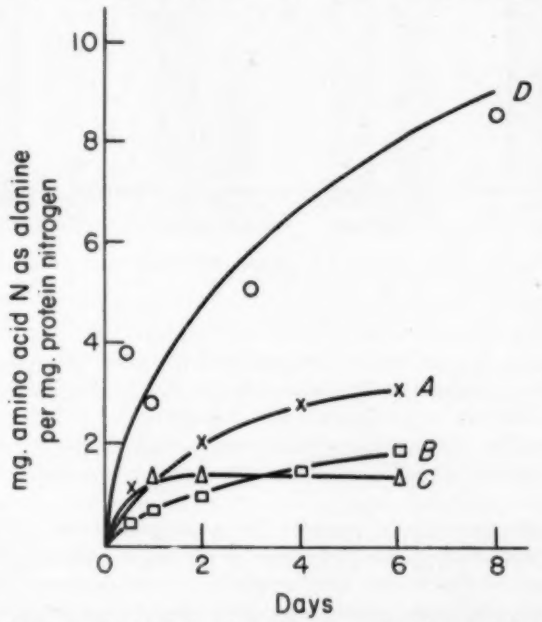


Fig. 2.—The rate of release of amino acids from native tissue proteins during in vitro anaerobic autolysis of normal and tuberculous lung tissue at pH 5.0 (0.2 M acetate buffer); *A*, *B*, and *C* as in Figure 2; *D*, inflammatory zone.

The presence of low enzyme concentration or of inhibitors are two other possible causes for the lower activity. As determined by micro-Kjeldahl analyses, 40 to 70% of the protein nitrogen is hydrolyzed during a six-day period.

B. Hydrolysis of Nucleic Acids During Autolysis in Vivo.—By extracting the material stored at —30 C. with 0.2 N $HClO_4$, at the original pH, without subjecting it to autolysis, we demonstrated the presence of DNA in discrete caseous tubercles (Table 1), and in the surrounding inflammatory zones (195 and 186 γ per 0.1 ml. of homogenate, respectively). The highest concentrations (305 γ) were found in the contents of completely softened tubercles; the lowest (161 γ per 0.1 ml.) was in normal lung tissue (Fig. 3).

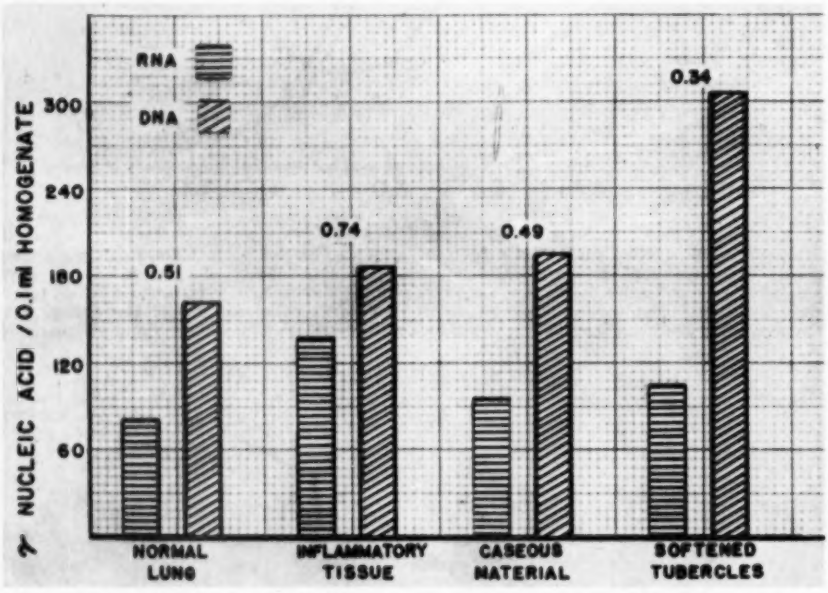


Fig. 3.—Concentrations of DNA and RNA in normal rabbit lung tissue and in various parts of a tubercle.

RNA was likewise found in the inflammatory zones (138 γ per 0.1 ml. of extract) and in the contents of caseous and softened tubercles (95 and 105 γ per 0.1 ml. of homogenate, respectively). These data are in harmony with the histochemical findings of Monaci and Koppitz [20] and of Bunting.[21] The latter remarked, "In tuberculous caseation, detectable desoxyribonucleic acids remain for what must be relatively long periods, of time, since they are found in the midst of caseous tissue."

Spectrophotometric analyses also revealed that in contrast to normal rabbit lung tissue, breakdown products of DNA and RNA are absent in homogenates of softened material and are present in only small amounts in extracts of caseous material (Fig. 4). Presumably, dialyzable hydrolytic products are lost from necrotic tissues.[22]

C. Hydrolysis of Nucleic Acid During Autolysis in Vitro.—Since DNA is an important constituent of the cell and its presence has been demonstrated in tuber-

culous caseous tissue, we studied the rate of hydrolysis of nucleic acids during anaerobic autolysis of lung tissue. As seen in Figures 5 and 6, homogenates of normal lung and those prepared from caseous tubercles show optimal DNase and RNase activities at pH 5.0. In the case of normal lung, the activity of both these enzymes continues beyond pH 7.0 without demonstrable peaks in the curve. A second optimal peak at pH 7.0 is seen in the curves for both RNA and DNA hydrolysis of caseous material.

The figures also show that "alkaline" DNase and "alkaline" RNase (most active at pH 7.0) still remain in the contents of softened tubercles at a time when "acid" DNase and "acid" RNase [23] (active at pH 5.0) have practically disappeared. Grogg and Pearse [24] published analogous observations on acid and alkaline phosphatase in guinea-pig tuberculous tissues. Weiss and Boyar-Manstein [1] have previously

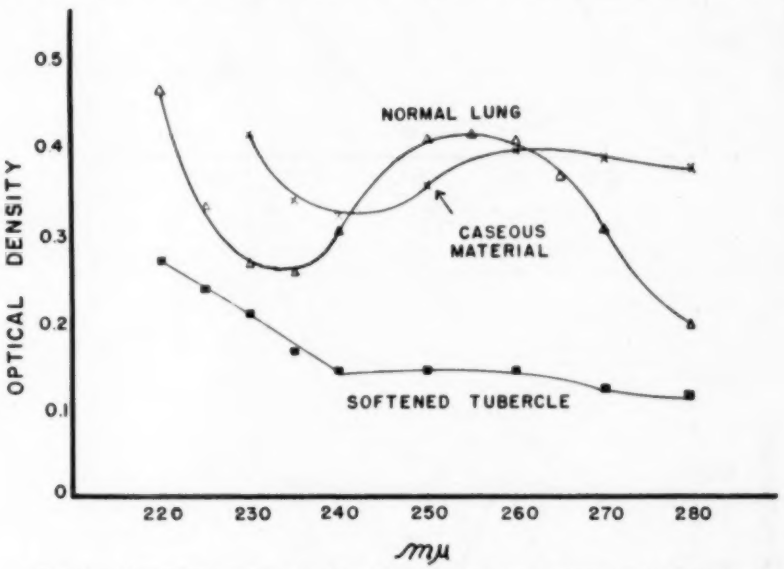


Fig. 4.—Absorption spectra of acid soluble fractions from normal and tuberculous lung tissue prior to autolysis, showing the absence of nucleic acid breakdown products in the contents of softened tubercles. 0.1 ml. of homogenate was extracted with 10 ml. of 5% $HClO_4$.

called attention to the fact that BA-amidase whose optimal activity is at pH 5.0 has completely disappeared from the contents of softened tubercles, while LA-peptidase (optimum, pH 7.8) still functions, albeit its activity is markedly reduced. The findings of Maver and Greco [25] also show a correlation between proteolytic and nuclease activities of cathepsin preparations. "The close correlation between the activating and inhibiting factors for the nuclease and proteolytic activities of these cathepsin preparations (tissue extracts) suggests either that they may be functions of a complex cathepsin molecule or that they are separate enzymes with very similar physical and chemical properties."

Very little work has been done on the subject of tissue ribonucleases. Roth [26] (1952), using rat kidney, observed one peak at pH 7.8 and a smaller one at pH 5.9, suggesting the presence of two enzymes.

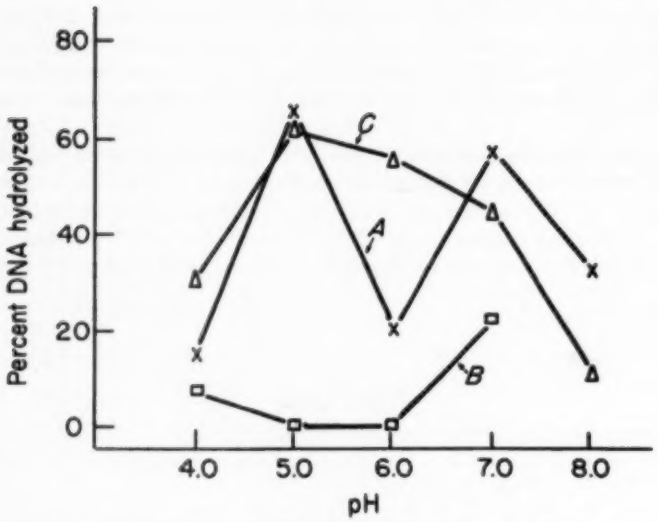


Fig. 5.—pH activity curves for in vitro anaerobic hydrolysis of DNA in normal and tuberculous lung tissue homogenates after 24-hour incubation at 35 C. *A*, *B*, *C*, as in Figure 2.

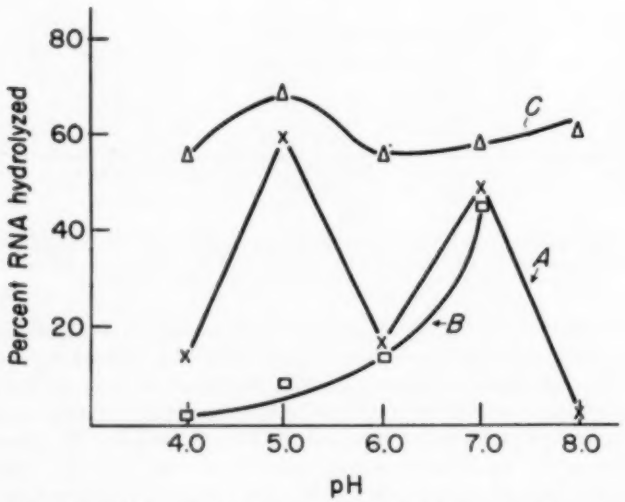


Fig. 6.—pH activity curves for in vitro anaerobic hydrolysis of RNA in normal and tuberculous lung tissue homogenates after 24-hour incubation at 35 C. *A*, *B*, *C*, as in Figure 2.

When tuberculous caseous material autolysed at pH 5.0, approximately 70 to 80% of the RNA and DNA were hydrolyzed during the first 48 hours (Figs. 7 and 8). During the next four days there was little further activity, probably because most of the substrate had been hydrolyzed. There was no evidence of any inhibitory action in vitro at this optimal pH. Homogenates of tissue from inflammatory zones autolyzed most rapidly, owing to abundance of endocellular nucleases. The creamy necrotic material, removed from completely softened tubercles, showed practically no DNase activity at pH 5.0. Hydrolysis of RNA proceeded slowly. There was about 50% loss of RNA at the end of the sixth day of observation.

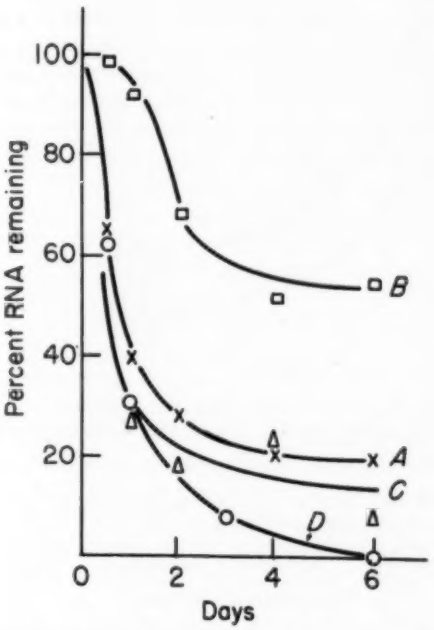


Fig. 7.—The rates of in vitro hydrolysis of RNA in normal and tuberculous lung tissue homogenates at pH 5.0 (0.2 M acetate buffer): *A*, *B*, *C*, as in Figures 2 and 3.

Little information is available in the literature on the chemistry of the autolysis of normal lung tissue. Figures 6 and 7 show that homogenates of fresh normal rabbit lung have strong RNase and DNase activities. It is also of interest to note that the ratio of RNA to DNA which we observed for normal rabbit lung is similar to that recorded by Schneider and Klug ‡ for normal rat lung. Moreover, our optimal pH for proteolysis agrees with the data of Onisi § on normal autolyzing rabbit lung and with older observations on autolyzing mammalian liver.

D. pH of Various Areas of Tubercles.—Hydrogen-ion concentration is one of the regulating factors in enzymatic activity. Since there is no report in the literature

‡ Reference 27, cited by Davidson, J. N. The Biochemistry of Nucleic Acids (Methuen's Monographs on Biochemical Subjects), New York, John Wiley & Sons, Inc., 1950.

§ References 28 to 30.

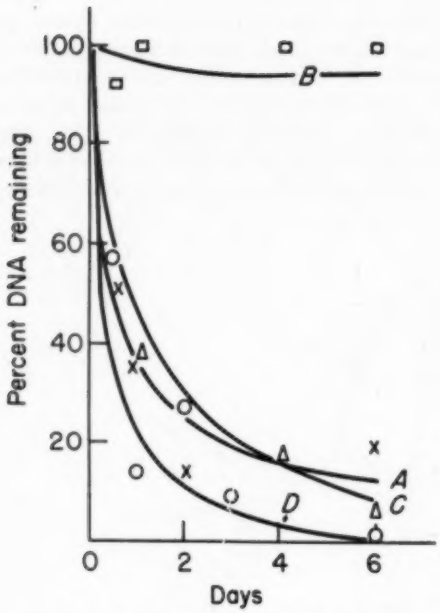


Fig. 8.—The rates of in vitro hydrolysis of DNA in normal and tuberculous lung tissue homogenates at pH 5.0 (0.2 M acetate buffer): *A*, *B*, *C*, as in Figures 2 and 3.

TABLE 2.—*pH of Homogenates Prepared from Various Areas of Lung Tubercles**

Rabbit	Centers of Caseous Tubercles	Adjacent Uninvolved Areas	Inflammatory Zones	Contents of Softened Tubercles	Walls of Softened Tubercles
	A. Early Lesions (5½ to 6 weeks)				
342	6.7	6.7-6.8	...	...	...
341	6.6-6.7	...	6.5-6.6	...	...
367	6.7	6.8	6.6	...	...
366	6.5-6.6	6.8	6.6	...	...
343	6.7	6.7-6.9	6.7-6.9	...	...
	B. Intermediate Lesions (9 to 10 weeks)				
356	6.4-6.5	...	...	...	...
347	6.4-6.5	6.9	6.8	...	...
365	...	6.6	6.4	7.0-7.1	...
W	...	6.5	...	7.2-7.3†	6.5
	C. Late Lesions (26 weeks)				
378	...	6.7	...	7.4	6.9
393	6.3‡	6.7	...	7.3-7.4	6.6

* Homogenates of lung from three healthy rabbits showed pH's of 7.0, 7.0 and 7.1 respectively.
† Contents of five softened tubercles, pooled.
‡ Lung infiltrated with caseous material.

on the pH of discrete, air-borne, experimentally induced tubercles, the pH data in Table 2 are of particular interest.

The animals were killed by exsanguination under ether anesthesia. The lungs were immediately removed and the tubercles or softened material dissected out. Homogenates were prepared at once in ice-cold 0.25M sucrose solution, and pH determinations made by means of the glass electrode. Since there are inherent difficulties in existing methods for measurement of intracellular pH (these have been reviewed by Spek),[31] we chose the sucrose homogenate methods as the best for determining the tissue pH. This method probably comes closest to indicating a combination of the extracellular and intracellular pH in a particular area.

In comparison with normal rabbit lung tissue, homogenates of which had a pH of 7.0 to 7.1, the pH of caseous material ranged from 6.4 to 6.7. These data must be interpreted in the light of the work of Papper [32] who showed that under ether anesthesia arterial blood has a pH of 7.0 instead of 7.4, owing to the accumulation of lactic acid. The inflammatory zones surrounding tubercles were found to be of pH 6.5 to 6.9. The contents of softened tubercles were more alkaline, being from pH 7.0 to 7.3 at 9 to 10 weeks and pH 7.4 or higher in animals killed 26 weeks after infection. This alkaline reaction may, in part, be due to the presence of basic amino acids and/or histones or the loss of acid phosphates or other diffusible ions.

The association of alkaline pH with tissue necrosis deserves further study. Schade and Claussen [33] (1925) found pH's of 7.1 to 7.2 in human cold abscesses. Rous [34] observed alkaline reactions in injured and devitalized experimental mouse tissue grafts. Gilding [35] observed similar results in liver cells killed by means of virulent hemolytic streptococci and in cartilage damaged in situ by freezing. Borger and Mayr [36] (1935) reported pH's up to 7.8 in rabbit kidneys subjected to infarction and necrosis. Knepper [37] (1937) found that the pH of most purulent exudates was acid but that of a human tuberculous lymph node was alkaline (pH 7.37). Since granulation tissue as well as necrotic tissue resulting from sensitization was alkaline (pH 6.95 to 8.31), he concluded that tuberculous tissue is alkaline because both sensitization and necrosis play a part in its pathogenesis.

COMMENT

The following questions have been raised: What is the source of the enzymes concerned in the softening of tubercles? Are they derived from cells of the inflammatory exudate or from tubercle bacilli? Is the pH favorable? Do inhibitors interfere with the progress of enzymatic processes?

Our investigations have been designed to answer some of these questions. It must be emphasized that the difficulty in obtaining adequate amounts of suitably controlled experimental material constitutes one of the roadblocks in the solution of this problem. It requires large numbers of animals to provide sufficient tissue for the isolation and purification of individual enzyme systems and inhibitors. We have, therefore, been obliged to work with tissue homogenates which contain the enzymes which may be involved in softening (BA-amidase, LA-peptidase, DNase and RNase) as well as others.

As to the question, are tubercle bacilli the source of the enzymes concerned in softening Lurie || has recently shown that in tubercles produced in rabbits by means

|| Personal communication to the authors.

of virulent bovine bacilli, with the aid of the Wells apparatus, the bacteria are numerous in the first stages of caseation but tend to disappear as the process advances. When softening is more or less complete, there are enormous numbers of tubercle bacilli. This phenomenon, first observed with human material, has led to the suggestion that these organisms may be the agents of softening. Our enzymatic results show however, that caseous material which has relatively few tubercle bacilli has good proteolytic activity, whereas the contents of softened tubercles which have a very high bacterial population, has little or no proteolytic or neucleolytic activity. This observation, together with the fact that cultures of Mycobacterium tuberculosis are poor in proteinase and nuclease activities,[38] makes it doubtful that tubercle bacilli per se are the source of the hydrolytic enzymes involved in softening. We can therefore tentatively conclude that the enzymes reponsible for the processes of softening (autolysis) of tubercles are released from injured phagocytic cells. A direct, experimental approach to this problem is, however, difficult.

There is suggestive evidence in the literature that tubercle bacilli may liberate a kinase (similar to streptokinase [39]) which activates tissue proteinases. Thus, Frahm, and co-workers [40] have shown that the proteinases of an emulsion of normal guinea-pig lung which ordinarily cannot hydrolyze casein, will be activated to do so, if the organ is macerated and inoculated in vitro with a living culture of tubercle bacilli.

Contrary to earlier workers, our in vitro results show that caseous material possesses sufficient enzymatic activity to digest approximately 60% of its own protein in six days and 70% of its nucleic acid in 24 hours. The in vivo changes which result in softening are slow and complex. It usually requires about 12 or more weeks to observe this phenomenon grossly in rabbits. There are several possibilities as to the cause of the in vivo delay in hydrolysis of "caseous" protein: 1. We have shown that the in vivo pH is unfavorable and permits only 25% of maximal proteolysis. 2. The proteolytic enzymes in the caseous area may require an active form of some co-enzyme or activator. These activators are either lacking or an unknown period of time must elapse to produce the low redox conditions necessary for their proper function. 3. There is present an inhibitor of peptidase activity [2] whose function may also be to retard proteolysis. 4. The proteins of the caseous tubercle may be resistant to rapid hydrolysis. 5. We have demonstrated decreased activity or disappearance of proteolytic enzymes from necrotic centers of the tubercle.[1] During homogenization we may bring enzymes from the outer layer of the tubercle in contact with necrotic tissue from the center, a process which may take months in vivo. 6. Under in vivo conditions, the proteolytic enzymes which are present in high concentrations in the inflammatory area may eventually penetrate the tubercle and softening may thus occur at an accelerated rate.

In contrast to the slow rate of proteolysis, 70% of the RNA and DNA are hydrolyzed in vitro in 24 hours. The buttery, softened material shows an accumulation of RNA and DNA but negligible RNase and DNase activity. It remains to be determined to what extent the nucleases are concerned in the process of softening. It is known that the tubercle has an impaired but functioning circulation ¶ and it is possible that a dynamic equilibrium exists in which nucleic acids are slowly hydrolyzed while new nuclear debris is being deposited. This equilibrium may also explain

¶ References 6 and 41.

the high protein content of the softened material. Perhaps softening ensues after only a small percentage of the protein is hydrolyzed, the remaining fibrous DNA giving the buttery material its viscous consistency.[42]

The delayed hydrolysis of nucleic acids in vivo cannot be attributed to unfavorable pH since we have shown two optima, one at pH 5.0 and another at pH 7.0, for DNase of caseous material. It may, however, be due to the presence of enzymatic inhibitors. Weiss and Singer [2] demonstrated a DNase inhibitor in vitro in caseous and in softened tubercles as well as in extracts of living virulent tubercle bacilli. Henstell [43] found such an inhibitor in bone marrow.

The appearance of softening probably involves more than the mere hydrolysis of a fibrous protein reticulum. There may be a gain or loss of cations (Na, K, Mg, Ca, etc.) as well as of anions, such as phosphate. This may explain the consistent alkaline pH of softened material. Basic amino acids or histones may also accumulate in the necrotic material. The cationic concentration in the caseous tubercles may play an additional role by influencing the activity of both the proteinases and the "acid" and "alkaline" nucleases (Miyaji and Greenstein).[44]

That low molecular weight compounds are lost by dialysis from caseous tubercles is illustrated in Figure 3 where one sees peaks at 260 mμ, indicating the presence of acid-soluble phosphorus compounds in normal lung and in caseous material, but a total lack of these derivatives of nucleic acid break-down in the contents of softened tubercles.

We plan to continue these studies on the chemical changes during the various stages of caseation and softening of tubercles. The following topics will be investigated: (*a*) proteins; soluble and insoluble; histones; protein N, antigenicity and electrophoretic mobility of these proteins; (*b*) amino acids; free amino acids; NPN; -S-S- and -S-H- compounds; (*c*) lipids, saponifiable and nonsaponifiable; (*d*) Nucleic acids, DNA, RNA, P, acid-soluble P, and inorganic P; (*e*) cationic concentrations of Ca, Mg, Mn, Na, and K; (*f*) Water content.

This information should aid our understanding of the chemical mechanisms most likely to explain the process of softening of tubercles.

SUMMARY AND CONCLUSIONS

By the application of enzymologic and spectrophotometric methods we showed that tissue removed from caseous and softened (necrotic) tubercles can undergo active autolysis under anaerobic conditions in vitro. Caseous material possesses sufficient enzymatic activity to hydrolyze approximately 60% of its own protein in six days and 70% of its nucleic acid in 24 hours.

The enzymes concerned in these processes are probably derived from broken-down phagocytic cells and not from the tubercle bacilli. This hypothesis is based upon the following data: (*a*) Tubercle bacilli are known to be low in number in the centers of caseous tubercles where we found proteolytic and nucleolytic enzymes to be quite active. (*b*) Tubercle bacilli are known to be present in enormous numbers in the contents of softened tubercles where we found enzymatic activity to be low or absent. (*c*) Tubercle bacilli have been shown by others to be poor in proteolytic and nucleolytic enzymes.

As to the cause of the delayed autolysis (softening) of caseous tubercles we have brought forth the following new evidence: While the optimum for hydrolysis of cellular proteins during autolysis is pH 5.0, in situ measurements with the glass

electrode show reactions of pH 6.4 to 6.7 in young tubercles. This range of pH permits only 25% of maximal protein hydrolysis in the course of six days' autolysis in vitro.

Tuberculous tissue contains significant amounts of RNA and DNA. The nucleic acids appear to accumulate in increased amounts within the contents of softened tubercles. Whether this material is derived from the accumulation of dead or dying phagocytic cells or whether the increase is due to a loss of water, remains to be determined.

It is likely that pH per se does not play a decisive role in regulating the hydrolysis of nucleic acids during autolysis, since both DNase and RNase show activity at both pH 5.0 and pH 7.0.

Spectrophotometric analyses have revealed that breakdown products of nucleic acid are absent from the contents of softened material, although present in normal lung tissue and in caseous tubercles. Presumably these products, as well as various cationic and other enzymatic activators, may be lost as the result of tissue injury.

An incidental new observation was made, namely, that the pH of necrotic lung tissue (the contents of softened tubercles) is consistently alkaline, being pH 7.4 or higher.

Dr. Herbert L. Ratcliffe, Director of the Penrose Research Laboratories, Zoological Society of Philadelphia, infected the rabbits.

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EXPERIMENTAL ASPIRATION PNEUMONIA

III. Pneumonia Produced by Intratracheal Injection of Carbohydrate Solutions

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DURING experiments on the production of acute pulmonary edema in rabbits by intratracheal injection of milk, feeding mixtures, and sugar solutions,[1] it was noted that animals surviving the initial injection of the material subsequently developed pneumonia. The pneumonia produced by injection of sugar solutions was similar in many respects to that produced by injection of milk [2] or solid foods.[3] The present report describing this pneumonia indicates that sugar solutions, even in low concentrations, cannot safely be substituted for milk in the management of infants with feeding problems.

METHODS

Intratracheal injections of varying concentrations of five carbohydrates (dextrose, lactose, maltose, dextrin, and fructose) and of several prepared feeding mixtures were given with sterile precautions to 36 healthy adult rabbits. Milk was not included in the feeding mixtures. The solutions, varying in strength from 6.25 to 50%, were injected into the trachea with the animals under ether anesthesia. Twelve of the animals died of acute pulmonary edema and were excluded from this study. The strength of the solution, the amount injected, and the fate of the remaining 24 animals after injection are recorded in the Table. The animals that did not die of pneumonia were killed at varying times after aspiration by intravenous air injection. At autopsy a minimum of four blocks of tissue, selected from both lungs, were fixed in 4% formaldehyde solution. Paraffin sections were stained by routine hematoxylin and eosin methods.

Seven control animals were given intratracheal injections of 5 cc. amounts of sterile distilled water, and three animals were given intratracheal injections of 5 cc. amounts of sterile 0.9% sodium chloride solution. These animals were killed in 24 hours (3), 48 hours (3), one week (3), and five weeks (1). Cultures were taken of four different parts of the lungs of each of the 10 control animals.

RESULTS

Five animals developed nonfatal pulmonary edema. These were killed at intervals of 30 minutes, 1 day (two animals), 14 days, and 59 days after injection. Four animals that had not developed pulmonary edema died at intervals of 4 days, 23 days (two animals), and 34 days. The remaining 15 animals were killed at intervals of one day to eight weeks after injection. At autopsy all 24 animals

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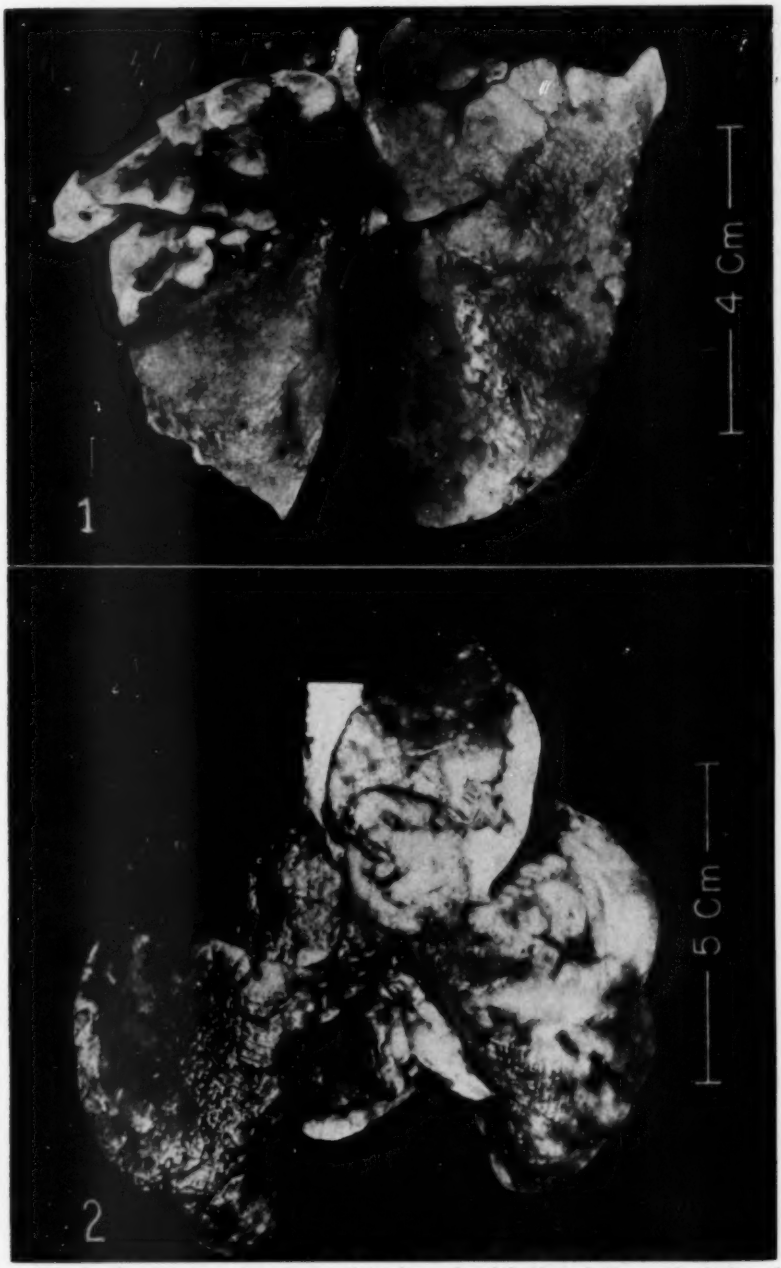

Fig. 1.—Pneumonia and empyema eight days after injection of 10 cc. of solution of Dextri-Maltose No. 1 and lactose in water.

Fig. 2.—Pneumonia, nodular granulomas, and emphysema 34 days after injection of 10 cc. of 12.5% dextrose.

showed pneumonia. The pneumonia varied considerably in severity, but the average reaction was not quite so intense as that elicited by injection of similar amounts of milk in earlier experiments.[2]

The gross findings were varied. Over one-half of the animals showed hemorrhagic pneumonia, collapse, and emphysema. These changes were most frequently seen in the dependent portions of the lungs. Three animals showed abscess formation, and two showed empyema, in addition to the pneumonia. Four animals had small irregular, but sharply defined, nodular areas suggestive of chronic granulomas. The lungs of 10 of the animals grossly showed only small areas of

Results of Injection of Various Carbohydrate Solutions

Material	Amount and Concentration of Solutions	Length of Survival After Injection	Autopsy Findings†
Dextrose	5 cc.; 50%	30 min.*	Pneumonia (early)
Dextrose	5 cc.; 50%	1 day*	Pneumonia
Dextrose	10 cc.; 12.5%	34 days	Pneumonia, abscess
Dextrose	20 cc.; 6.25%	5 days*	Pneumonia
Dextrose	10 cc.; 6.25%	4 days	Pneumonia
Lactose	10 cc.; 25%	43 days*	Pneumonia, granulomas
Lactose	20 cc.; 12.5%	14 days*	Pneumonia
Lactose	10 cc.; 12.5%	26 days*	Pneumonia, granulomas
Lactose	20 cc.; 6.25%	14 days*	Pneumonia (slight)
Lactose	10 cc.; 6.25%	29 days*	Pneumonia, granulomas
Maltose	10 cc.; 25%	30 days*	Pneumonia, abscess
Maltose	20 cc.; 12.5%	59 days*	Pneumonia, granulomas
Maltose	10 cc.; 12.5%	6 days*	Pneumonia
Maltose	10 cc.; 6.25%	6 days*	Pneumonia
Dextrin	20 cc.; 25%	1 day*	Pneumonia
Dextrin	10 cc.; 25%	13 days*	Pneumonia
Dextrin	20 cc.; 12.5%	23 days	Pneumonia, abscess, empyema
Dextrin	10 cc.; 12.5%	13 days*	Pneumonia
Dextrin	20 cc.; 6.25%	23 days	Pneumonia
Dextrin	10 cc.; 6.25%	1 day*	Pneumonia (slight)
Fructose	20 cc.; 12.5%	4 days*	Pneumonia
Dextri-Maltose	20 cc.; 15 gms./100 cc.	1 day*	Pneumonia
Dextri-Maltose, lactose	10 cc.; D.-M. 7.5 gm., lactose 5 gm./100 cc.	8 days*	Pneumonia, empyema
Lactum	20 cc.; 5.0%	2 days*	Pneumonia

* Animals were killed.

† Pneumonia was diagnosed by either gross or microscopic examination. The complications listed were all seen grossly.

emphysema or collapse, and the diagnosis of pneumonia was not made until the microscopic examinations. None of the 10 animals given injections of sterile distilled water or 0.9% sodium chloride showed gross changes.

The early microscopic changes varied from slight alveolar thickening with scanty patchy pneumonic areas to extensive pneumonia with abscess formation, collapse, and emphysema. The reaction in the first 24 hours was made up primarily of granulocytes and red blood cells in the alveolar spaces. Occasional monocytes were seen in the alveoli and in the interstitial tissues. Within the first week the exudate consisted of granulocytes and monocytes, with monocytes predominating in many areas. Large interstitial collections of epithelioid cells with early granulomas were found, and there was marked thickening of many alveolar walls. Occasional foreign body giant cells were seen.

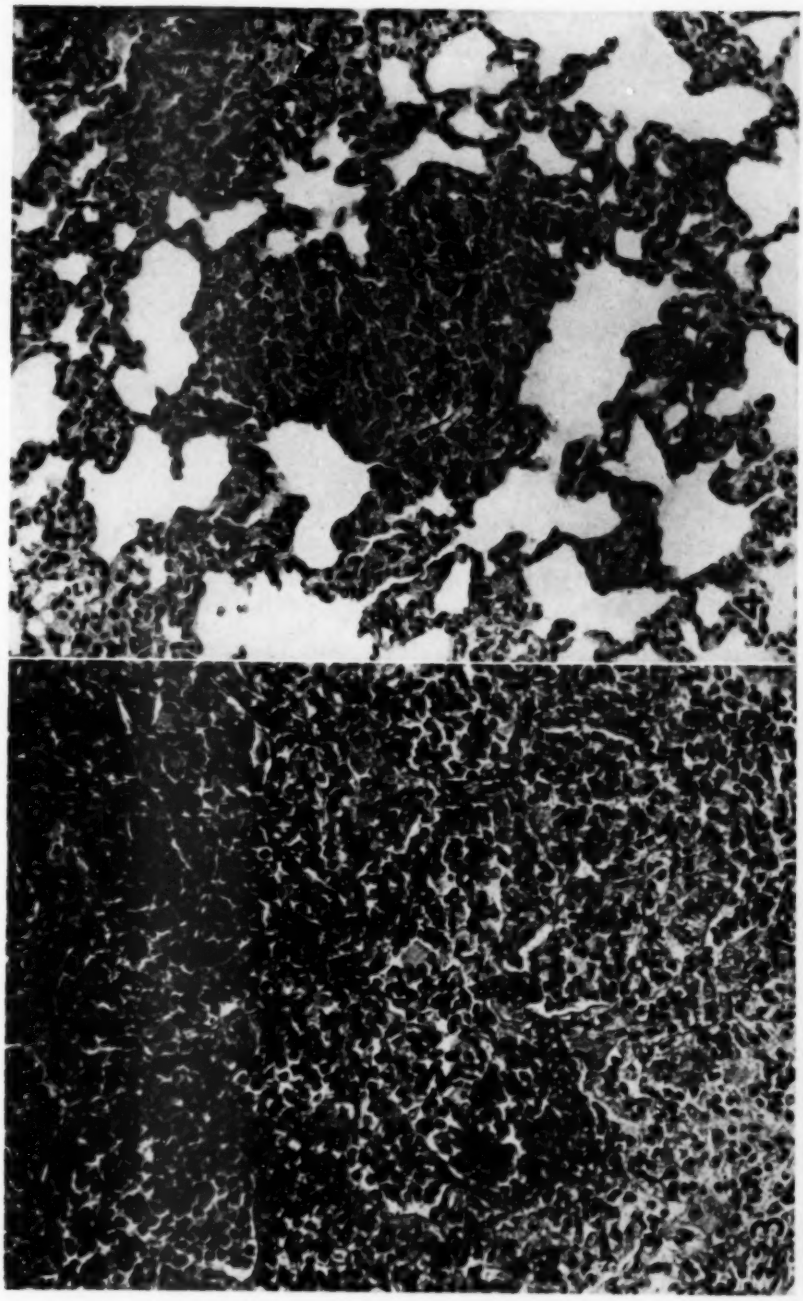

Fig. 3.—Pneumonia 24 hours after injection of 10 cc. of 25% dextrin. Granulocytes and red blood cells predominate; × 275.
Fig. 4.—Focal granuloma, alveolar thickening, and emphysema 14 days after injection of 20 cc. of 12.5% lactose; × 275.

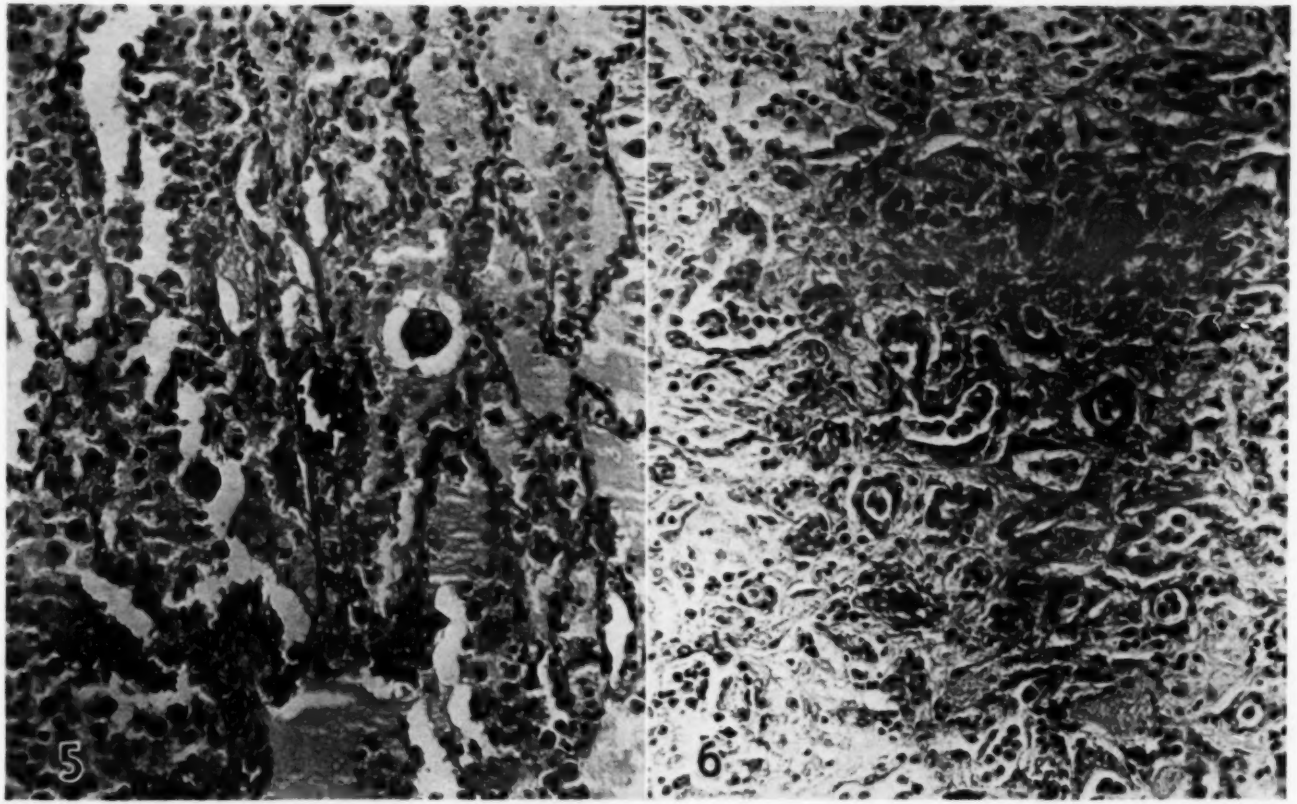

Fig. 5.—Mononuclear pneumonia with giant cell reaction three weeks after injection of 20 cc. of 12.5% dextrin; × 275.

Fig. 6.—Mononuclear pneumonia with cuboidal appearance of alveolar walls. Animal died 34 days after injection of 10 cc. of 12.5% dextrose; × 275.

In two to four weeks the cellular reaction was chiefly mononuclear, unless abscess formation had occurred. At this time alveolar thickening and chronic granulomatous reaction was prominent, and in some areas fibrosis had occurred. Many alveoli showed an appearance resembling cuboidal epithelium, and in some areas these alveoli were grouped together, giving an adenomatous appearance to the tissue. Giant cell reaction was prominent in several instances. Emphysema and collapse were also found. At five to eight weeks most of the acute reaction had disappeared, but slight to moderate alveolar thickening and scanty mononuclear pneumonic areas were still present. Interstitial fibrosis with organization of the exudate, emphysema, and collapse, was seen in one animal killed at eight weeks.

The lungs of four of the seven control animals given injections of sterile distilled water showed no significant microscopic changes; two presented mild patchy pneumonia, and one animal had a moderately severe pneumonia. A small amount of pink-staining unidentified foreign material was found in this animal. The lungs of one rabbit injected with sterile saline showed no significant changes. In two animals given injections of sterile saline a scanty patchy pneumonic reaction was present, but the reaction was not comparable to that observed in the animals receiving sugar solutions.

All four cultures were negative at the end of 72 hours in each of four control rabbits. Three control rabbits had three negative cultures and one positive culture; two rabbits had two positive and two negative cultures, and one rabbit had one negative and three positive cultures. The organisms most frequently recovered were nonhemolytic staphylococci and coliform bacilli.

COMMENT

The production of acute pulmonary edema in rabbits by intratracheal injection of carbohydrate solutions has previously been described.[1] Carbohydrate solutions of 12.5% or greater consistently produced acute pulmonary edema and death when injected in 20 cc. amounts; whereas concentrations of 25 or 50% were required to produce pulmonary edema with 10 cc. amounts. Ten or 20 cc. amounts of 6.25% solutions did not cause pulmonary edema. However, the findings reported here indicate that intratracheal carbohydrate injections may produce pneumonia, even when dilute solutions are used and pulmonary edema is not produced.

The principal clinical application of these findings lies in the field of pediatrics, where oral or gavage feeding of carbohydrate solutions is common. Many pediatricians caring for premature babies or other infants with feeding problems have substituted sugar solutions for milk in the hope of preventing aspiration pneumonia. The production of aspiration pneumonia as reported here and the previous report of the production of acute pulmonary edema by strong sugar solutions suggest that this feeding procedure is dangerous.

SUMMARY

The production of aspiration pneumonia in rabbits by intratracheal injection of carbohydrate solutions and feeding mixtures containing no milk has been described. The reactions to the injected sugar solutions included acute pneumonia with abcess

formation and empyema and mononuclear pneumonia with chronic granulomatous reaction, fibrosis, collapse, and emphysema. The pneumonia is similar to, but usually not so severe as, that produced by injection of similar amounts of milk.

The practice of many pediatricians of substituting carbohydrate solutions for milk in the care of newborn infants with feeding problems would seem hazardous in view of the previously reported production of acute pulmonary edema in rabbits by intratracheal injection of strong carbohydrate solutions and the findings reported here of aspiration pneumonia caused by dilute sugar solutions.

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FURTHER CYTOCHEMICAL STUDIES IN SYSTEMIC LUPUS ERYTHEMATOSUS

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KLEMPERER, Gueft, Lee, Leuchtenberger, and Pollister,[1] in 1950, reported on the frequency of hematoxylin bodies in tissues of patients who died of systemic lupus erythematosus. Their Table 1 recorded the incidence of hematoxylin bodies in 35 cases of systemic lupus erythematosus. Although in 32 of the total of 35 these bodies were found, they were absent in 3, and only in 24 cases were they seen in more than one site. Since it was concluded that these changes were the result of a disturbance of nucleic acid metabolism affecting mesenchymal cells, it could have been expected that such lesions should be encountered without exception and widely distributed throughout the body. The scarcity and occasional absence of hematoxylin bodies was accounted for by inadequacy of the available material, because in several instances there were only a limited number of slides in the files of the laboratory. It seemed, therefore, desirable to examine new material without such limitations, but also to repeat the histochemical analysis of the hematoxylin bodies, and to add certain new techniques which might strengthen and amplify previous conclusions. Moreover, the new material revealed certain variations of the hematoxylin bodies which had not been fully explored in the preceding report. These observations led to questions regarding the relationship between the hematoxylin bodies and the "fibrinoid" changes of the connective tissue in systemic lupus erythematosus which demanded clarification.

MATERIAL AND METHODS

Since the original report of Klemperer and his associates,[1] 14 cases of lupus erythematosus have come to autopsy. Every one of these cases presented clinical symptoms which justified the diagnosis of systemic lupus erythematosus. This clinical diagnosis was supported in seven cases by the detection of L. E. cells in bone marrow aspirates and in many instances by the ability of the patients' sera to provoke the formation of L. E. cells if incubated with normal white cells (L. E. phenomenon). This study was carried out during a period in which a consistent search for L. E. cells had not been established on the medical service. Four of the patients had received corticotropin (ACTH) and cortisone therapy. However, in only three of them, A15329, A15496,

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A14952, was the treatment continued over a period of more than a year, while the other patients received hormone therapy (Corticotropin) for only a short period of days to a few weeks. Tissue blocks were taken from all the viscera except the brain, where limitation of autopsy permit prevented examination. They were fixed in neutral formalin solution (formaldehyde P. P. and distilled water 1:5), Zenker-Formalin, and Carnoy's solution (3 parts absolute alcohol and 1 part glacial acetic acid). As mentioned in a previous publication, for the demonstration of hematoxylin bodies formalin-fixed material is preferable to Zenker-Formol because the bodies appear more distinct in hematoxylin-eosin-stained sections. Many connective tissue staining methods and a multiplicity of appropriate histochemical techniques were used. Uniform 5 μ paraffin sections of Carnoy-fixed material were used for all the histochemical studies. The Feulgen reaction was done according to the method recommended by Stowell.[2] Control unhydrolyzed preparations were made routinely. The periodic acid-Schiff reagent (PAS) method was done after McManus' procedure.[3] Ultraviolet absorption spectra were determined with a Beckman quartz monochromator, a Beckman hydrogen arc lamp, and a 1P28 photomultiplier followed by a direct coupled amplifier and microammeter. The maximum sensitivity used was 5×10^{-9} amperes full scale photomultiplier current. Slit widths varied from 0.1 to 0.6 mm., depending on the amount of energy available. A Bausch and Lomb Polaroir Design V 0.72 N. A. reflecting-refracting objective was used, with a similar condenser. The areas scanned were kept constant at about 5 by 20 μ in the determination of a single absorption spectrum. Spectra were determined in triplicate on the same area, then averaged for the spectrum shown in the figures. The Millon and residual protein methods are those of Pollister and Mirsky,[4] and we are indebted to the former for much assistance and information.[5]

Photometric measurements of Feulgen and Millon reactions were made with an apparatus similar to that of Pollister and Moses [6] but with the Beckman monochromator at a slit width of 0.2 mm. Trypsin digestions were done with crystalline trypsin (Armour) in phosphate buffer at pH 7.3, used in 5, 10, and 20 mg. per milliliter quantities, at 37 C. for 4 to 16 hours.

MORPHOLOGIC OBSERVATIONS

The Libman-Sacks type of endocardial involvement was observed in four cases (31%) in this series. This compares well with the observations of Gross [7] and Klemperer, Pollack, and Baehr 30%).[8] Generalized lymph node enlargement was recorded in 11 of this series, while it occurred only 9 times in 20 cases previously reported. This discrepancy is probably due to its receiving more attention in the records in our series. The frequency of the characteristic lymph node necrosis was only slightly higher (33%) than in the previous series, where it was noted in 25%.

In discussing the microscopic observations of this series, such findings will first be commented upon which we do not consider as of diagnostic significance, then those which we believe to be significant though not absolutely characteristic, and finally those which we regard as specific for the disease.

The occurrence of focal myositis and myolysis has been mentioned briefly previously,[8] but no emphasis was given to these findings because of the paucity of observations. The frequency of such lesions in this series deserves attention only because it supports the belief of Sokoloff and co-workers [10] that nodular infiltrations of the striated muscle are not characteristic of rheumatoid arthritis (Steiner and Chason [11]). The pathogenetic factors responsible for such alterations and the rare myolysis remain obscure.

Special attention was paid to establish the frequency of plasma cell proliferation, because of the report of Rosahn and Fox,[12] who found innumerable plasma cells in the lymph nodes of one of their patients. In view of persistent statements in the literature of a background of hypersensitivity in systemic lupus erythematosus, it appeared necessary to establish the frequency of conspicuous plasma cell proliferation in lymph nodes and spleen, since Teilum [13] had correlated allergic tissue changes

with plasmocytosis and had also considered the fibrinoid changes in systemic lupus, as well as the glomerular wire loops and periarterial splenic fibrosis, as morphologic equivalents of hypersensitivity. It should be mentioned here, however, that Teilum did not find conspicuous plasma cell proliferation in his cases of systemic lupus erythematosus. We observed plasma cells in lymph nodes and spleen in seven cases, and in one of these in very conspicuous numbers. In five of these cases this was associated with decubital ulcers or other secondary infections. In view of this coincidence, and the absence of plasma cells in half our cases, we believe that a pathogenetic correlation would be highly speculative.

To the second group of histologic alterations belong the onion peel periarterial fibrosis of the spleen; the deposits of "fibrinoid" material in various localizations, including wire loops, and hyalin thrombi. The microscopic increase in metachromatic ground substance, as seen within the pericardium and myocardial septa, has not been included, because such microscopic increments are also seen in other morbid

TABLE 1.—*Frequency and Distribution of Hematoxylin Bodies in Fourteen Consecutive Autopsies*

Autopsy No.	Kidney	Heart	Nodes	Ovary	Spleen	Pleura	Pancreas	Uterus	Sk. Muscle	Tube	Esophagus	Liver	Skin	Breast	Stomach	Pylorus	Intestine	Adrenal	Periadrenal Tissue	Marrow	Bladder	Testis	Tongue	Gallbladder	Vagina	Pneumonia or Infection	Age, Yr.	Sex	
14305	+	..	+	+	..	+	+	..	..	..	..	+	..	..	..	..	..	..	+	..	..	..	..	..	..	+	23	F	14305
14380	+	+	+	..	..	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	40	M	14380
14384	+	..	..	+	+	+	..	..	+	+	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	26	F	14384
14399	+	+	+	+	+	+	..	+	..	..	..	..	+	..	..	..	+	..	..	..	..	..	..	..	..	+	45	F	14399
14416	+	+	+	+	+	..	..	+	..	+	..	..	+	..	..	+	..	..	..	..	..	..	..	..	..	+	25	F	14416
14417	+	+	+	+	+	..	..	..	..	..	..	+	+	..	+	..	..	+	+	+	..	..	..	+	..	+	43	F	14417
14420	+	+	+	..	..	..	..	+	+	+	+	..	..	..	..	..	+	..	..	..	..	..	..	..	..	+	13	F	14420
14423	+	+	+	+	..	..	+	..	+	..	..	..	..	..	+	+	..	..	..	..	..	..	+	..	..	+	31	F	14423
14549	+	+	+	..	..	..	+	+	..	..	+	..	..	..	..	..	..	..	..	+	..	+	..	..	..	+	16	M	14549
14566	+	+	+	+	+	+	+	+	+	..	..	+	..	+	..	..	..	+	..	..	+	..	..	..	..	..	13	F	14566
14576	+	+	..	+	+	..	..	..	..	..	..	..	..	+	..	..	..	..	..	..	..	..	..	..	+	+	22	F	14576
14952	+	+	+	..	..	+	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	14	M	14952
15329*	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	23	F	15329
15496	+	+	..	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	28	F	15496

* L. E. cells found during life.

states, such as rheumatic fever. The quality of the former lesions did not differ materially from the observations recorded in previous articles. However, the frequency of the occurrence of glomerular wire loops and hyalin thrombi was higher (100%), while periarterial splenic fibrosis was found in seven cases only (60%), in contrast to 95% reported previously.

As mentioned in the introduction, it is one of the purposes of this report to reevaluate the validity of the hematoxylin bodies as a specific criterion for the autopsy diagnosis of systemic lupus erythematosus. Table 1 shows that in a series of 14 consecutive cases these lesions were present in all, with one exception. This exceptional case, however, showed L. E. cells in bone marrow and blood during life. Hematoxylin bodies were found in at least three different sites. A study of Table 1 reveals that they are found in certain organs with particular frequency: kidney, ovaries, lymph nodes, and heart. These organs are, therefore, most adequate for the study of control material. We selected the ovaries and kidneys for such investigations because of the easy detectability of hematoxylin bodies in these sites. Results

of such studies are reliable only if one is assured that equal investigative intensity has been applied to both sources of material. Obviously a responsible investigator will always fear to have unconsciously lacked in objectivity in examining control material. For this reason we applied the following technique. We established, for the ovaries, two minutes as the maximal time necessary for the unquestionable recognition of a sufficient number of hematoxylin bodies, using the oil-immersion lens. Fifty control cases, including 8 instances of necrotizing polyarteritis nodosa, were searched for a period of 15 minutes each. In the examination of kidney sections we ascertained that in most cases of systemic lupus erythematosus hematoxylin bodies were found in practically every field. In two cases the search was successful only after 20 glomeruli had been examined with the high-power lens. Consequently,

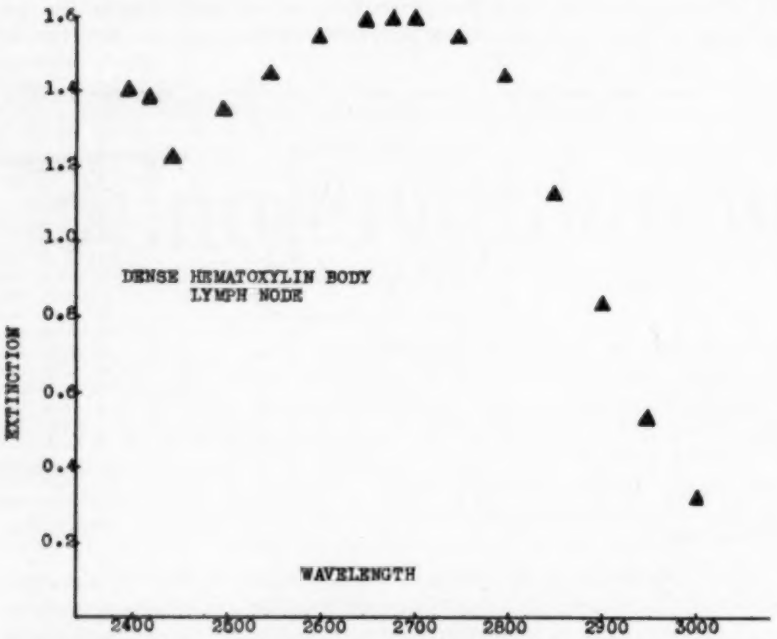

Fig. 1.—Ultraviolet absorption curve of hematoxylin-body aggregates in lymph nodes.

we scrutinized 60 glomeruli in the control cases before we discontinued further search for hematoxylin bodies. With the application of this method we were unable to find hematoxylin bodies in any one of the control cases.

The observation of these bodies in all except 1 of 14 consecutive cases of this series, and their absence in 50 most carefully examined control cases, and the results of the preceding investigation of Klemperer, Gueft, Lee, Leuchtenberger, and Pollister, in 1950,[1] give the assurance that free hematoxylin bodies are found only in systemic lupus erythematosus. These elements, therefore, constitute a reliable criterion for the diagnosis in autopsy material.

The next section describes our further histochemical observations.

Study of deeply stained hematoxylin body aggregates, as seen in lymph nodes and endocardial vegetations (Figures 5 and 6 of Reference 1), had shown that a considerable amount of desoxyribose nucleic acid was present. This identification was accomplished by a controlled Feulgen reaction and the evidence of marked absorption of ultraviolet light at 2,537 A.[1] It seemed desirable to confirm these results by a study of the absorption of ultraviolet light of varying wave length. A curve was obtained (Fig. 1) which shows a broad peak at 2,600 to 2,700 A. consistent with nucleoprotein. The usual nonspecific absorption below 2,500 A. is seen.

Hot trichloracetic acid treatment removed the positive Feulgen reaction and the high ultraviolet light absorption, as had been shown in 1950. Preliminary studies

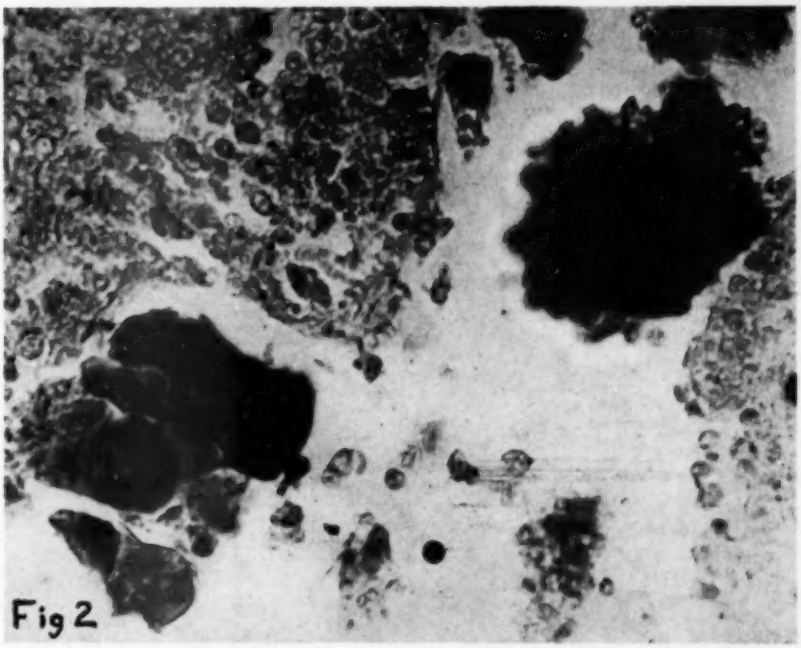

Fig. 2 (Case 14416).—Lymph node showing Millon reaction. Wratten C4 Filter (Blue Separation Filter); × 400. Intense coloration of large hematoxylin bodies is striking. Nuclei and red cells are somewhat stained.

were made, comparing the optical density of the bodies and normal lymphocytic nuclei both by ultraviolet absorption of 2650 A. and in Feulgen-stained sections. The optical densities of the ultraviolet measurements of the bodies and lymphocytic nuclei agreed within our limits of error. However, the Feulgen-reacting material of the bodies appeared diminished compared to lymphocyte nuclei. It is possible that absorbing nucleotides are measured in the ultraviolet measurement, while they do not give the Feulgen reaction. In view of the paucity of data and of the complexity of the underlying problem, these observations are recorded without attempt at final interpretation. The Millon reaction was strongly positive before and after treatment with hot trichloracetic acid (Fig. 2).[14] Trypsin digestion at pH 7.3 caused

a significant decrease in the intensity of the Millon reaction but did not influence the visual intensity of the Feulgen reaction. A similar observation has been made upon normal lymphocyte nuclei in sections. The PAS technique stained the hematoxylin bodies conspicuously red before and after treatment with hot trichloracetic acid, trypsin digestion, and hot trichloracetic acid followed by trypsin digestion, as well as after treatment with saliva. Pectinase, lysozyme, and polygalacturonidase digestion did not affect the staining.[18] After incineration at 600 C. there was no appreciable residue.

These results permit the following conclusions: Desoxyribose nucleic acid (DNA) is present in the bodies. The DNA content measured in the Feulgen preparations varies considerably (Table 2). Analogous variations are noted in the intensity of hematoxylin staining of these bodies in routine hematoxylin and eosin

TABLE 2.—*Absorption of Feulgen-Stained Hematoxylin Bodies of Lymph Nodes, Measured at 5,460 A., 40 A. Effective Band Width, 10 μ Areas Measured, Arranged by Values of Optical Density*

No.	Optical Density
1	0.13
2	0.14
3	0.15
4	0.17
5	0.22
6	0.23
7	0.24
8	0.24
9	0.29
10	0.29
11	0.32
12	0.37
13	0.37
14	0.38
15	0.38
16	0.38
17	0.42
18	0.43
19	0.50
20	0.56
Average	0.31

preparations. In the most intensely stained hematoxylin bodies there is a higher concentration of DNA than in normal lymphocyte nuclei. In Fig. 1 the peak density of the body is 1.6, while nuclei at most are 0.9. Protein is present in considerable quantities. It is a type of nonhistone or residual protein not influenced by hot trichloracetic acid (using the sulfuric acid Millon reaction [14]), but it is digested by trypsin. Conversely, trypsin does not affect the DNA. The PAS stain reveals the presence of an admittedly obscure substance capable of coupling with Schiff's reagent after periodic acid oxidation. The substance is not glycogen. Since the results of incineration were negative, it might be worthwhile to reiterate the negative results of conventional stains for calcium and iron.

Further study of the hematoxylin-body aggregates in conventional hematoxylin-eosin-stained sections disclosed rather striking variations in the intensity of hematoxylin staining and Feulgen reaction of the bodies (Figs. 3 and 4). Some aggregates showed a mottling of hematoxylin-eosin staining; others were even completely

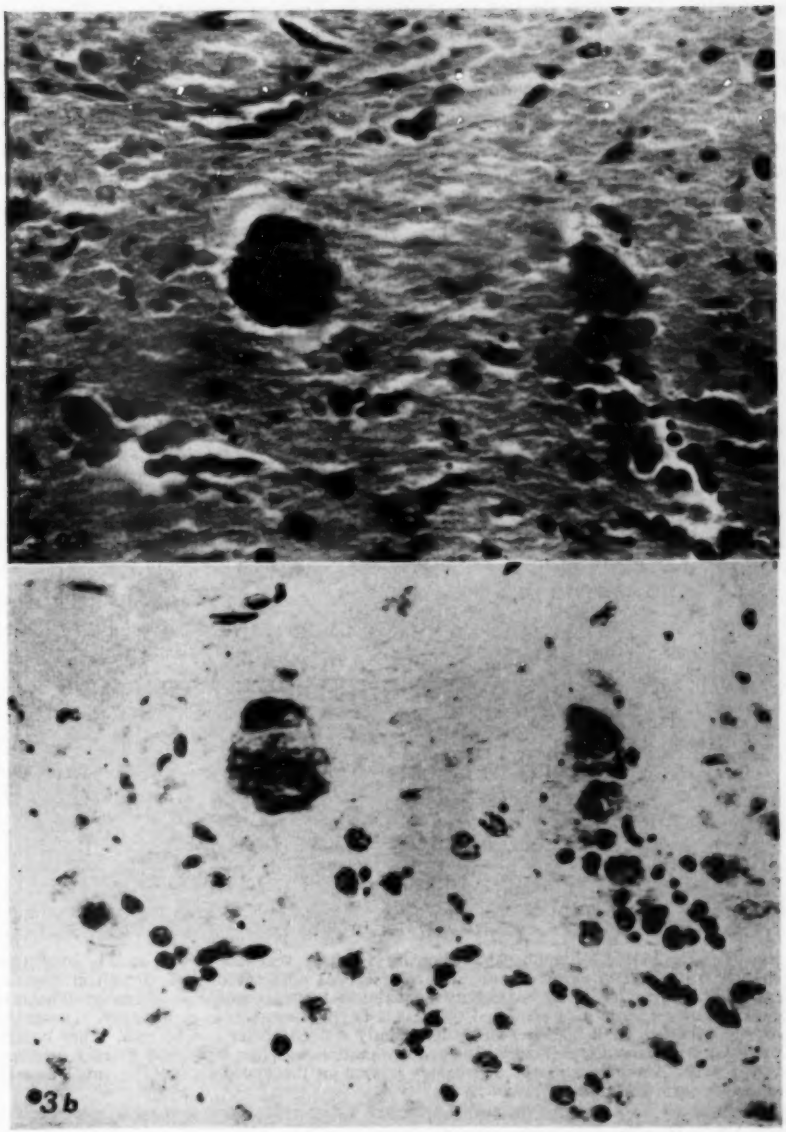

Fig. 3 (Case 14416).—*A*, lymph node. Hematoxylin and eosin; × 600; Wratten B filter (green). A hematoxylin body aggregate left of center composed of clumps varying in shade from black to gray. To the right of center a smaller darker-stained aggregate with four dark karyorrhectic particles at its lower edge. Scattered throughout the gray-stained eosinophilic matrix are darker and paler-stained structures, some of which have a distinct chromatin network. *B*, the same. Wratten A filter (red). The difference in shades is much accentuated and the intensity of staining of the clumps reduced because the eosinophilic component is abolished. The eosinophilic matrix seen in Figure 3*A* as gray is hardly visible, and the contrast between the dark-staining nuclei, pyknotic nuclear debris, and the smudged small hematoxylin bodies is more striking than in Figure 3*A*.

eosinophilic. The latter showed no staining in Feulgen preparations. Photometric measurements of 20 bodies in Feulgen-stained slides showed a great variation of DNA content, as high as four to one (Table 2). The Millon reaction for tyrosine-tryptophane-containing protein was still positive in all these feebler stained aggregates, as identified from serial sections stained by hematoxylin and eosin, Feulgen, and Millon techniques. It thus becomes possible to interpret such bodies as chemical compounds containing nucleoprotein in which the nucleic acid component has gradually diminished until, finally, it has completely vanished as far as one may judge by the histochemical methods available. In 1950, the first stage of this degradation was described as nucleic acid depolymerization.[1]

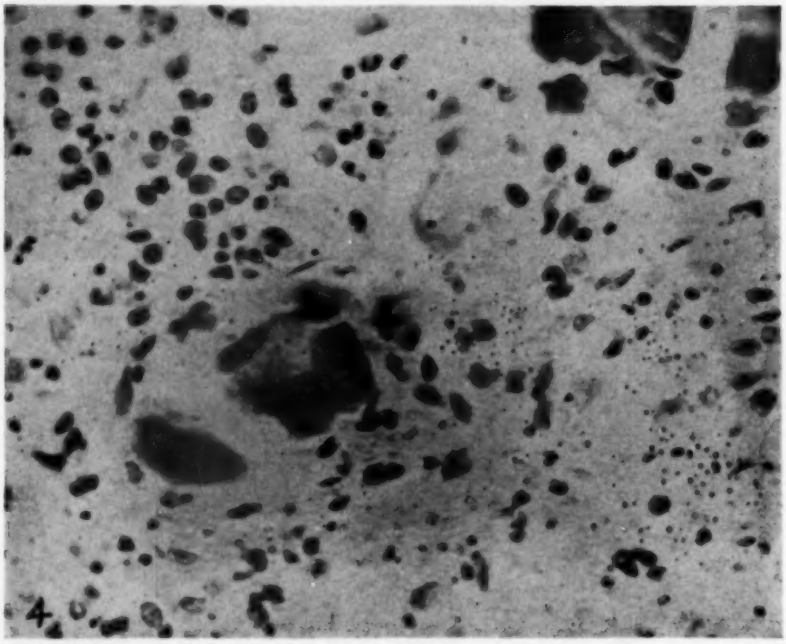

Fig. 4 (Case 14416).—Lymph node showing Feulgen reaction. Wratten 74 filter (pure green); × 600. Several of the aggregates from sections adjacent to those shown in Figure 3. The smaller aggregate is stained heavily and almost homogeneously in intensity. The other aggregate shows a large pale area, and the rest is fairly homogeneous in intensity. Toward the upper right several small homogeneously but faintly stained bodies can be seen. They contrast by their faint staining as well as by their fuzzy outline with the dark and sharply delineated nuclear debris. Another group of aggregates is seen in the upper right. The small dust-like particles are formalin pigment granules.

The fact that trypsin strikingly diminished the intensity of the Millon reaction, while hot trichloracetic acid did not affect it, shows that the action of these two reagents upon the hematoxylin bodies seems identical with that upon the normal nucleus. This would indicate that the protein constitution of the hematoxylin body does not differ from that of the normal nucleus and would suggest that the destructive nuclear process in lupus is centered not upon the proteins but mainly upon the nucleic acid and its probably weak linkage to basic proteins. In other words, from

this evidence, trypsin-like action upon the proteins of the nucleus is probably not operating in this disease. This allows us to ask again if a desoxyribonuclease (DNAse) system is affected in lupus, as previously suggested by Klemperer and his associates, in 1950. Serum DNAse is not increased * (Kurnick and co-workers [15]). DNAse inhibitors have recently been discovered in polymorphonuclear leucocytes, possibly within other hematic cells, by Henstell and Friedman [16] and by Kurnick and co-workers.[17] However, inactivation of these inhibitors by the L. E. factor could not be demonstrated by well-designed experiments.†

On the other hand, the positive PAS reaction in the bodies denotes the presence of a reacting substance, the origin of which is difficult to ascertain. Leuchtenberger and Schrader [19] and Schrader and Leuchtenberger,[20] in a study of a hemipteran egg, have suggested that PAS-positive material may originate from the DNA of the cell in its secretory activity. A similar origin of the PAS-positive material in the hematoxylin bodies cannot seriously be considered, because the PAS reaction is not affected by treatment with hot trichloracetic acid, which does, however, affect the nucleic acid and nucleotides contained within the bodies. Should one altogether entertain the idea that the PAS-reacting substance derives from nuclear chromatin? According to our present information, there is no evidence of any PAS-positive material in the normal nucleus, either from in vitro studies or cytochemical analysis. The only evidence which lets us think of the possibility that reactive aldehydes or glycols may, under the pathologic condition of the disease process, be derived from the protein moiety of the nuclear chromatin is our observation that normal lymphocyte nuclei show slight PAS positivity after hot trichloracetic acid treatment followed by trypsin digestion, whereas acid alone has no such effect. But the possibility must not be neglected that the appearance of PAS-positive material is no part of the progressive process of the particular chromatin degradation, but that the substance is incorporated from an extracellular source, for instance, from the connective tissue ground substance. These questions cannot be answered at the present time, but the observation of PAS-positive material does not invalidate the conclusions drawn from the DNA-protein studies that the hematoxylin bodies contain nuclear protein varying in DNA composition.

These observations and interpretations of the tinctorial variation of the hematoxylin bodies from hematoxylin affinity to eosinophilia directed our attention to the eosinophilic material commonly seen in lupus, the "hyalin thrombi" and "wire loops" of the glomeruli. Careful scrutiny of numerous "wire loops" and "hyalin thrombi" in hematoxylin-eosin-stained sections showed irregular tinges of purple and blue in many of these lesions (Fig. 5). In certain instances the "hyalin thrombus" appeared as a bluish plug (Fig. 6), while in some glomeruli the "wire loop" had a delicate purple smudge (Fig. 7). Feulgen preparations showed that many of the "hyalin thrombi" and "wire loops" were quite colorless, but an appreciable number had a faint positive reddish tinge, denoting the presence of desoxyribose nucleic acid (Fig. 8). Unhydrolyzed Feulgen stains showed no color. Karyorrhexis and karyolysis of the usual sort were also seen in such glomerular lesions. The products of the latter type of nuclear breakdown appeared as sharply defined deep blue particles (Fig. 9) and were not smudgy and purple-red like the hematoxylin bodies. Feulgen

* Dr. Maclyn McCarty made DNAse determinations on the sera of two patients with lupus.

† Lee, S. L.: Personal communication to the authors.

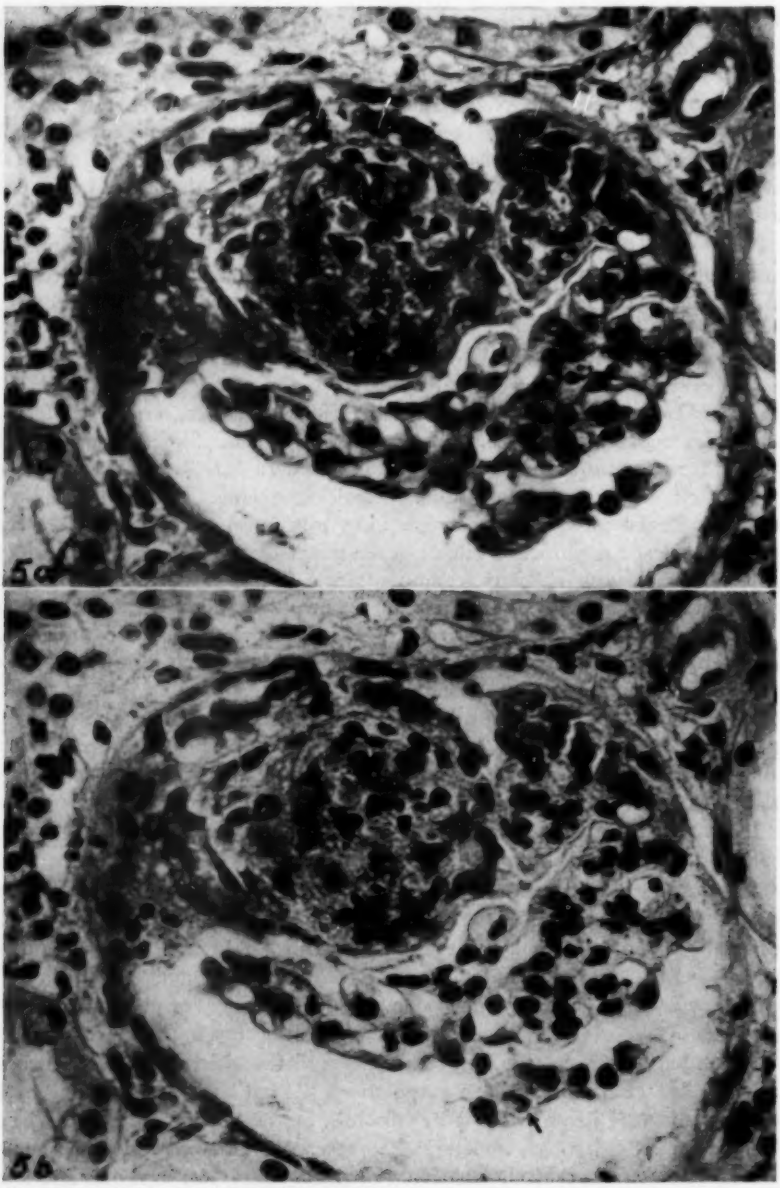

Fig. 5 (Case 14576).—*A*, Glomerulus. Hematoxylin and eosin; × 600; Wratten B filter (green). Glomerular capillary structure partly well-preserved. In the lowest glomerular capillary to the right of an epithelial cell there is a small hematoxylin body within the capillary lumen. The capillary structure of the upper half of the glomerulus is obliterated. Between 2 and 3 o'clock of the right upper field a frayed basement membrane can still be discerned, but the several hematoxylin-stained bodies cannot be definitely located within a capillary lumen. They may lie in an intermembranous space. The central portion of the glomerulus shows complete disorganization of capillary structure. Numerous faintly stained hematoxylin bodies are intermingled with proliferated cells of the mesangium, with an eosinophilic granular matrix. At the left there is an ill-defined eosinophilic band. *B*, the same, Wratten A filter (red). Hematoxylin-stained bodies show up much paler than in Figure 5*A*, indicating their large eosinophilic component.

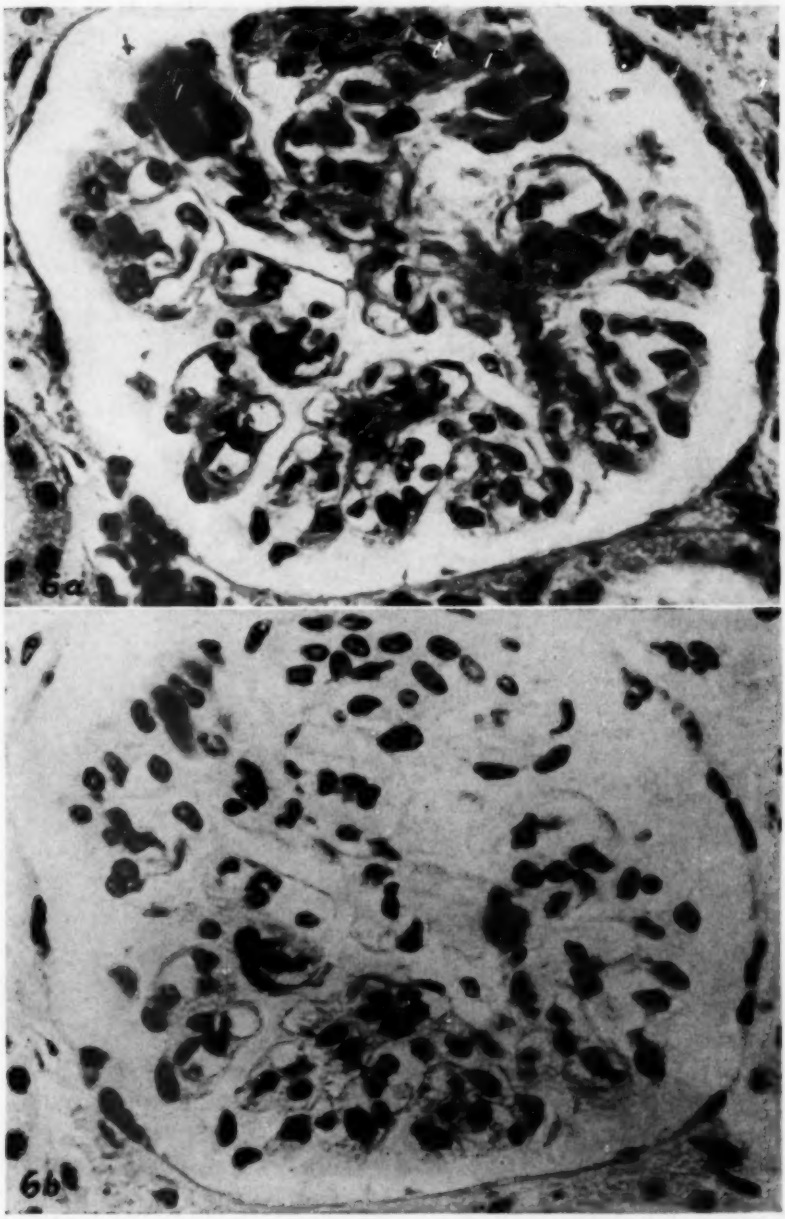

Fig. 6 (Case 14416).—*A*, kidney. Hematoxylin and eosin; × 600; Wratten B filter (green). Glomerulus with several hyalin thrombi at 11 o'clock, two of which stain deeply with hematoxylin, while the others are entirely eosinophilic and stain paler. *B*, same field as above. Wratten A filter (red). The hematoxylin-stained hyalin thrombi stand out conspicuously, while the eosinophilic ones are blocked out.

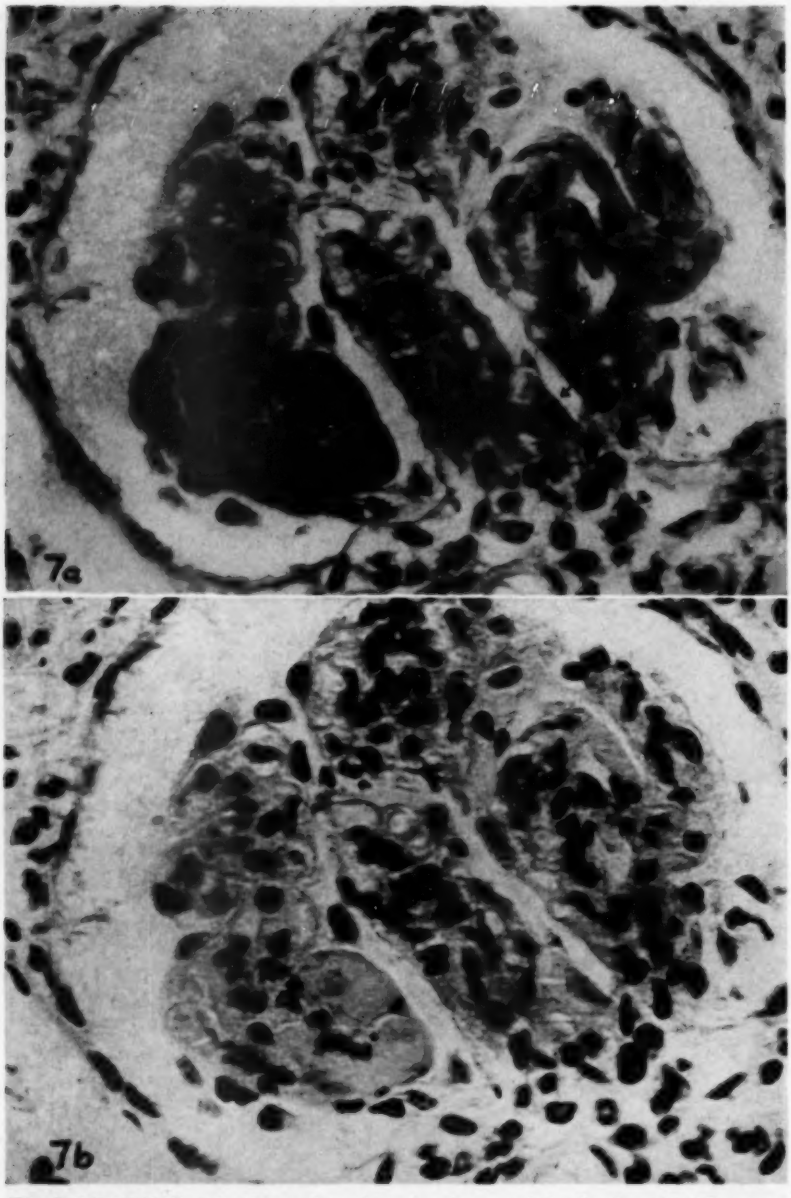

Fig. 7 (Case 14416).—*A*, glomerulus with wire loops and hyalin thrombi. Hematoxylin and eosin; × 600; Wratten B filter (green). The wire loops and hyalin thrombi on the left side blend with each other, and the basophilic smudges cannot be clearly localised. In the center of the glomerulus, left of a cleft separating two lobules running from south-east to north-west, there is a fragment of a wire loop showing basophilic smudging. The wire loop on the right side shows no such smudging. *B*, same glomerulus photographed with red filter (Wratten A) to accentuate basophilic areas. Note the difference between the two wire loops described above.

preparations revealed an analogous difference, in that the karyorrhectic particles were sharply defined and the hematoxylin bodies were smudgy and diffuse. This haziness cannot be removed by focusing, in contrast to that of karyorrhectic particles. Giemsa preparations show comparable color and structural differences. Because of the observation of an association of the smudgy type of degraded nuclear material with eosinophilic masses, we are inclined to interpret the "wire loops" and "hyalin thrombi" as containing end-products of nuclear breakdown of the type characteristic of systemic lupus erythematosus. Application of the histochemical methods of analysis described for the hematoxylin bodies of the lymph nodes supported this interpretation. The faint smudgy Feulgen reaction in the "wire loops" and "hyalin

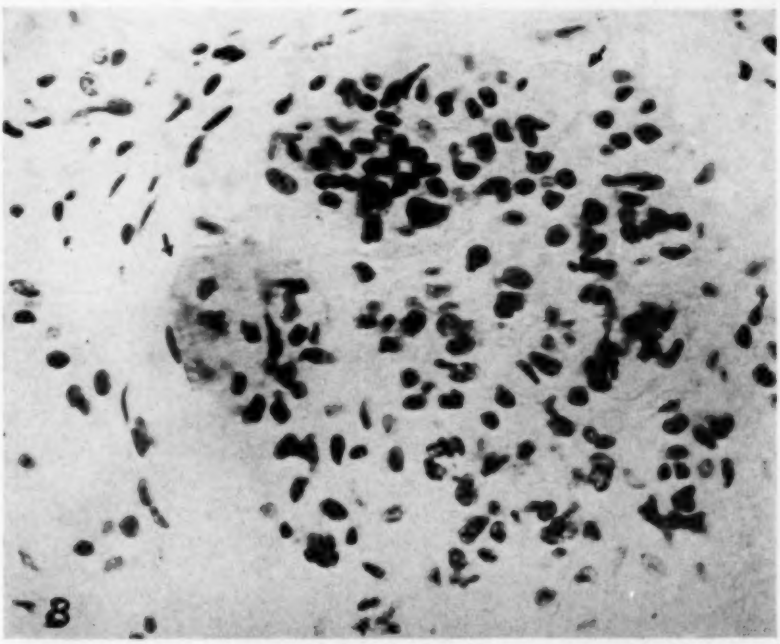

Fig. 8 (Case 14416).—Glomerulus. Feulgen reaction; × 600. Wratten 74 filter (pure green). Glomerulus with wire loop and hyalin thrombus. The hyalin thrombus at 1 o'clock is entirely unstained. The wire loop at the left, at 9 o'clock, shows faint but distinct smudged Feulgen positivity.

thrombi" was abolished by preceding treatment of the sections with hot trichloracetic acid. They gave a strongly positive Millon reaction (Fig. 10) and were easily digested by crystalline trypsin at pH 7.3. They stained yellow, like basic proteins, with an orange-G-aniline-blue mixture at pH 3.‡ The PAS reaction was strongly positive before and after hot trichloracetic acid treatment.

Ultraviolet absorption spectra of three thick "wire loop" lesions were determined to ascertain if the amount of nucleic acid sometimes noted in the Feulgen preparations was sufficient to show at the 2,600 A. peak for nucleic acid. No nuclei were

‡ References 21 and 22.

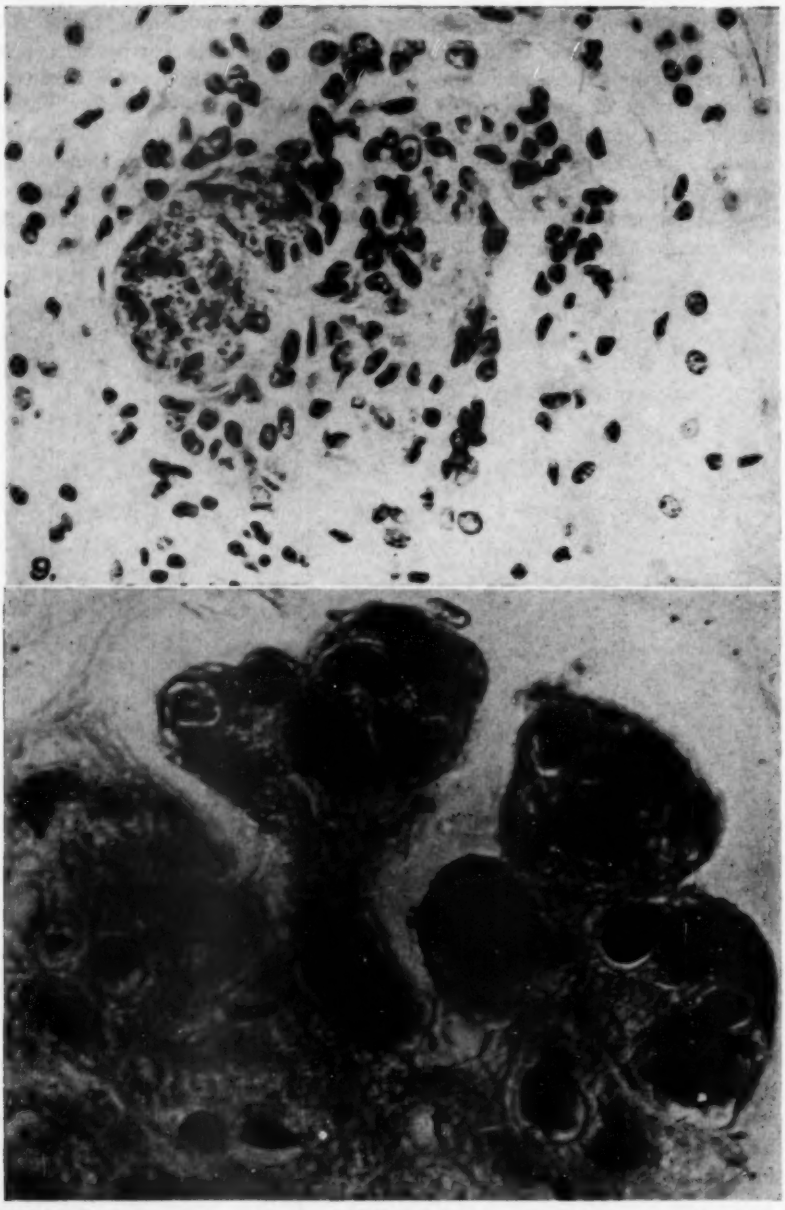

Fig. 9 (Case 14576).—Glomerulus. Hematoxylin and eosin; × 600; Wratten A filter (red). Shows the ill-defined faintly staining hematoxylin bodies, the eosinophilic component having been blocked out. In contrast, the deeply blue karyorrhectic nuclear fragments are sharply outlined and deeply stained.

Fig. 10 (Case 14416).—Glomerulus. Millon stain, photographed at 3,650 A. with Bausch and Lomb reflecting microscope, N. A. 0.72; approximately × 1,200. Hyalin thrombi and wire loops show strong light absorption, denoting heavy deposits of tyrosine-tryptophane-containing protein.

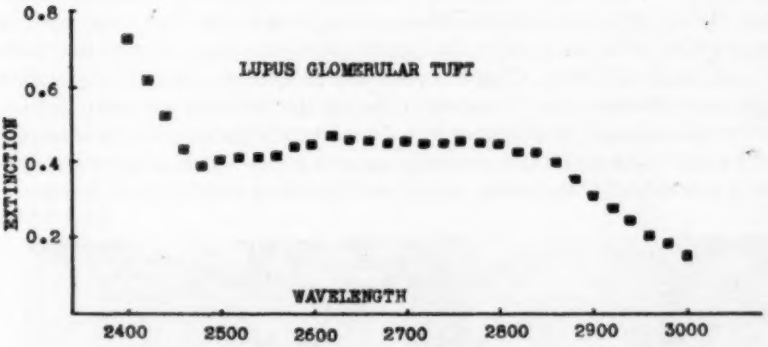

Fig. 11.—Ultraviolet absorption curve of glomerular wire loop.

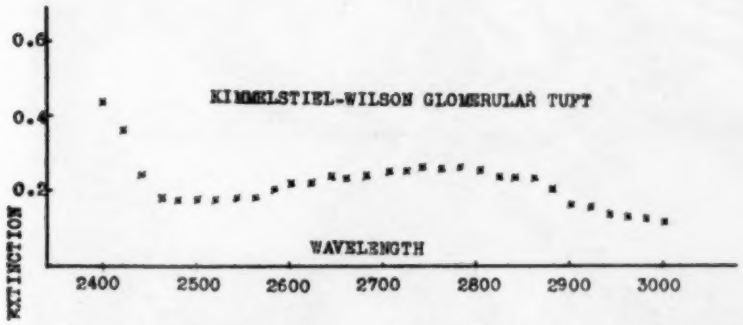

Fig. 12.—Ultraviolet absorption curve of a Kimmelstiel-Wilson glomerular lesion.

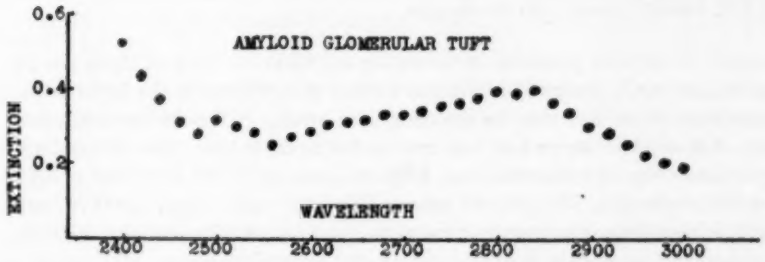

Fig. 13.—Ultraviolet absorption curve of an amyloid glomerulus.

seen in the areas investigated. Slit widths were constant at 0.6 mm. The area was about 5 by 20 μ. In one of these three lesions the curve showed a moderate rise in the 2,600 to 2,700 A. region. The curve is shown in Figure 11 and represents averages of three successive determinations on the same lesion. This single positive finding, together with the positive Feulgen reaction sometimes seen in the "wire loops" and "hyalin thrombi" (Fig. 8), confirms the evidence suggested by studies of the hematoxylin-eosin-stained sections (Fig. 6), that DNA is sometimes present in the "'wire loops" and "hyalin thrombi." To meet the objection that the moderate ultraviolet absorption at 2,600 A. might be present in morphologically similar conditions, we determined absorption spectra of the Kimmelstiel-Wilson lesions of

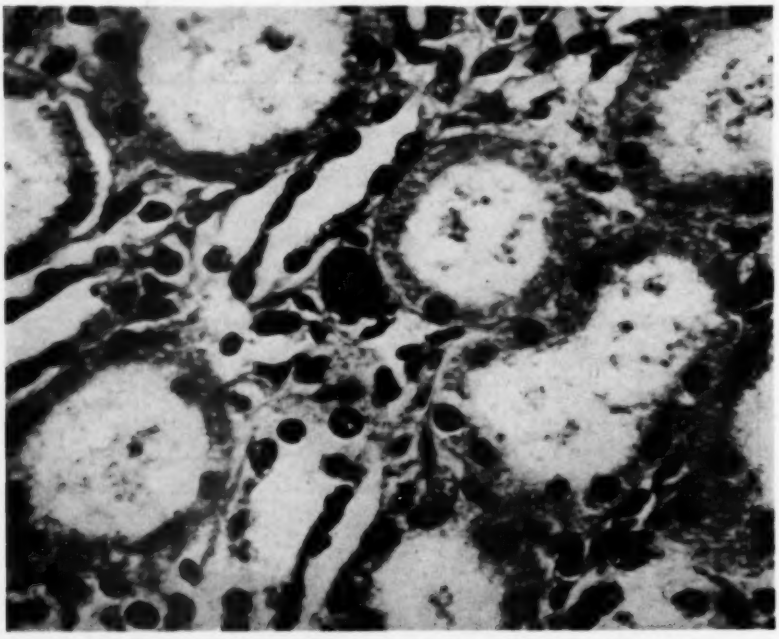

Fig. 14 (Case 14416).—Kidney. Wratten B filter (green); × 600. Intertubular capillary, showing body intensely stained with hematoxylin.

diabetes and of amyloid glomeruli in secondary amyloidosis. Both of these glomerular lesions, especially the amyloid, have a distinct resemblance to the lupus lesions in conventional stains and may be confused with lupus. It should be emphasized, however, that amyloid stains are negative in the lupus kidney. The Kimmelstiel-Wilson lesions (Fig. 12) showed a low, 2,800 A., peak, probably indicative of cyclic amino acids in proteins. The amyloid lesions (Fig. 13) had a high, 2,800 A., peak analogous in significance to that just mentioned and an additional slight, 2,500 A., peak, which has previously not been reported and the significance of which is unknown. However, both of these absorption spectra showed no rise at the 2,600 A. peak of nucleic acids. The 2,500 A. peak of amyloid is being studied further by the chemical separation of amyloid constituents, as previously demonstrated by Hass.[23]

Apart from the histochemical analysis of the eosinophilic "wire loops," the conventional staining technics, Weigert's fibrin stain and Mallory's trichrome and phosphotungstic acid hematoxylin, stain them like fibrin. Mainly because of this tinctorial behavior, the "wire loop" lesions were regarded as part of the systemic fibrinoid connective tissue alteration, which is so conspicuous a feature of the histologic picture of systemic lupus erythematosus.[8] The histochemical analyses suggest that the fibrinoid glomerular alteration in systemic lupus erythematosus might be determined by deposition of degraded nucleoprotein. Churg and Grishman [24] have recently demonstrated by phase-contrast microscopy of "wire loops" that a refractile homogeneous material is deposited between the epithelial and endothelial basement membranes.

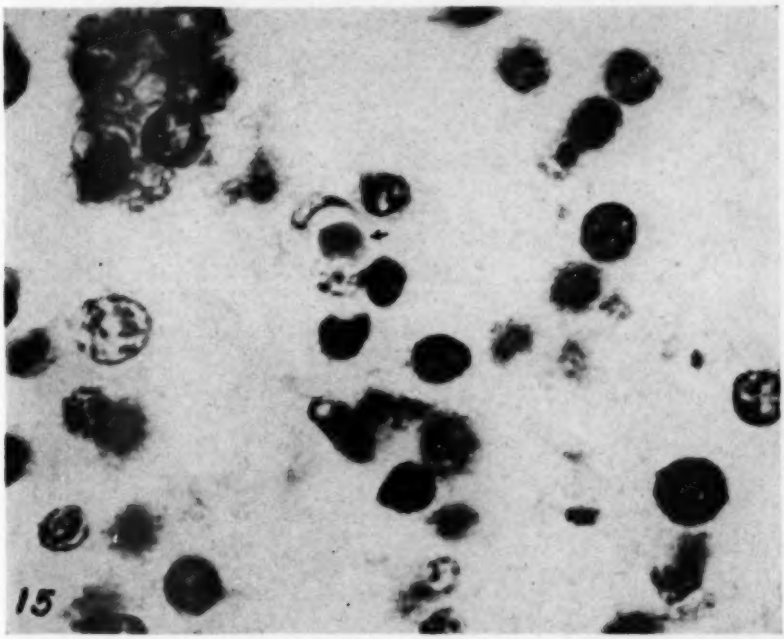

Fig. 15 (Case 14416).—Pancreatic island. Wratten A filter (red); × 1,200. Capillary containing a small hematoxylin body.

Intraluminal masses identical with those in the glomeruli were observed in the renal intertubular and pancreatic capillaries (Figs. 14 and 15). In addition, even larger vessels were seen, both of the greater and lesser circulation, which contained hematoxylin bodies either free or embedded in a granular eosinophilic matrix, occasionally revealed only by an ill-defined smudge (Fig. 16*A* and *B*). Such intraluminal aggregations were most probably not of embolic origin, because no source of embolisation was seen on careful gross and microscopic examination of the endocardium. Since these larger intravascular deposits are structurally and tinctorially identical with the intracapillary "hyalin thrombi," we regard them as degraded nucleoprotein circulating in the blood stream.

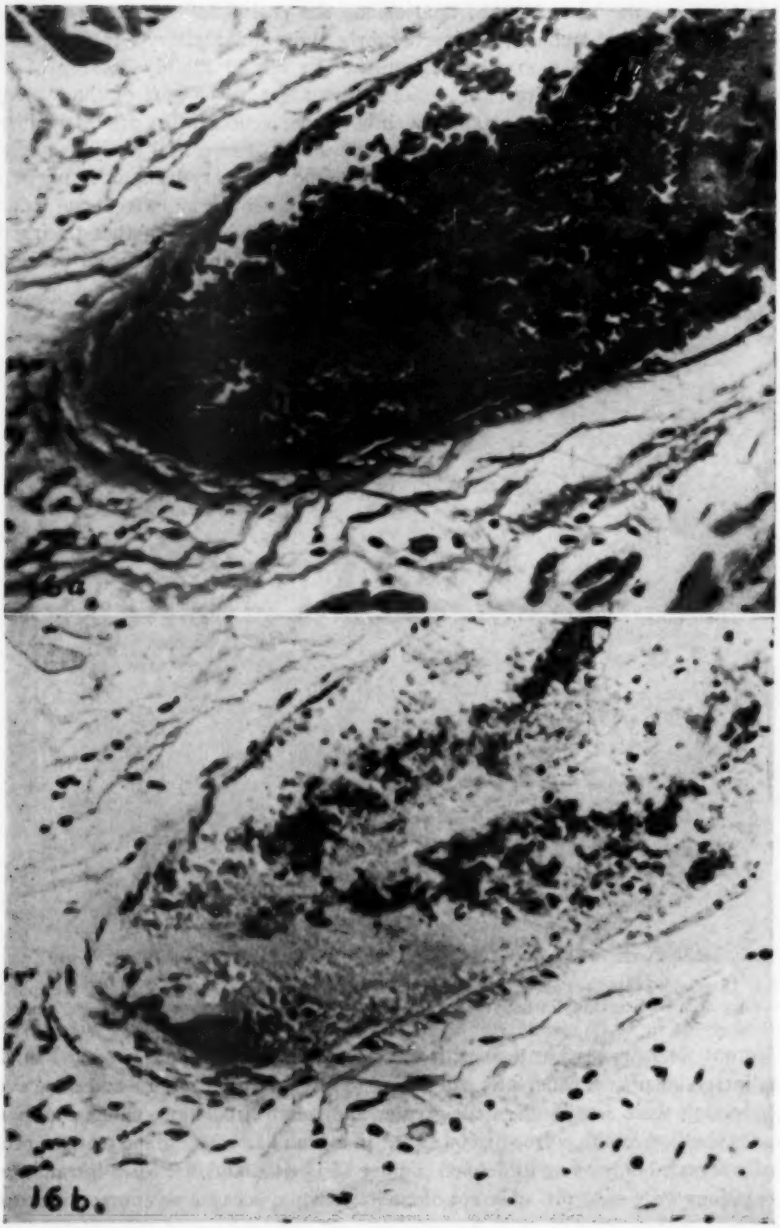

Fig. 16 (Case 14566).—*A*, myocardium. Hematoxylin and eosin; × 300; Wratten B filter (green). A dilated septal arteriole with a plug (no endocardial vegetation) formed by eosinophilic granular material within which there are many hematoxylin bodies embedded. The free lumen of the vessel contains red cells. *B*, the same. Wratten A filter (red). The hematoxylin bodies stand out conspicuously.

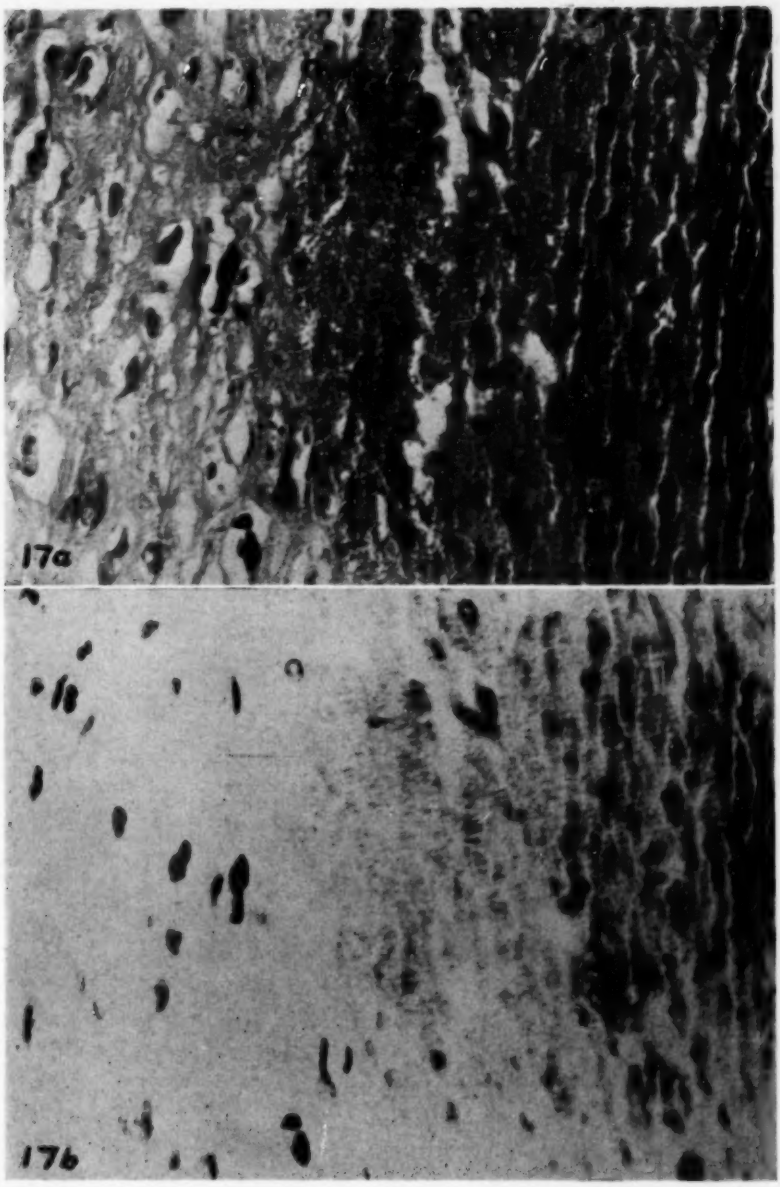

Fig. 17 (Case 14549).—*A*, connective tissue at base of mitral valve. Hematoxylin and eosin; × 600; Wratten B filter (green). Left side of figure shows closely packed stout collagen bundles separated by so-called tissue spaces, which contain fibroblasts. In the center, collagen bundles are stouter, beaded and granular, and, above all, stain more intensely. Fibroblasts can still be recognized. Further right, the bundles become very dark. *B*, the same. Wratten A filter (red). Connective tissue bundles at the left invisible. The course of bundles in the center and at the right are outlined by dark granules and elongated clumps deposited upon them.

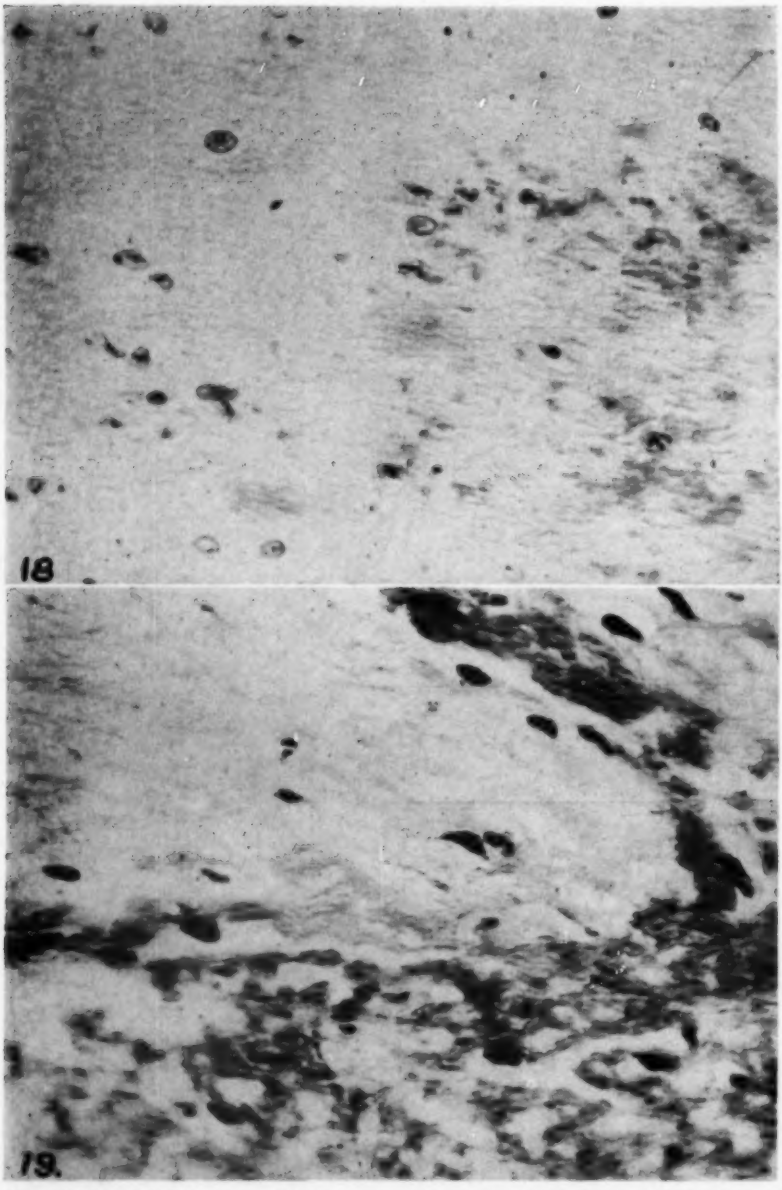

Fig. 18 (Case 14549).—Connective tissue at the base of the mitral valve. Feulgen reaction. Wratten 74 filter (pure green); × 600. Within an ill-defined unstained fibrillar matrix numerous indistinct faintly Feulgen-positive bodies. Cells in the vicinity show distinct chromatin pattern.

Fig. 19 (Case 14549).—Mural endocardium left ventricle. Hematoxylin and eosin; × 600; Wratten B filter (green). Center shows faintly stained wavy collagen fibers and a few fibroblasts. Periphery shows an irregular network of refractile stout darker stained (eosinophilic) bundles of fibrinoid.

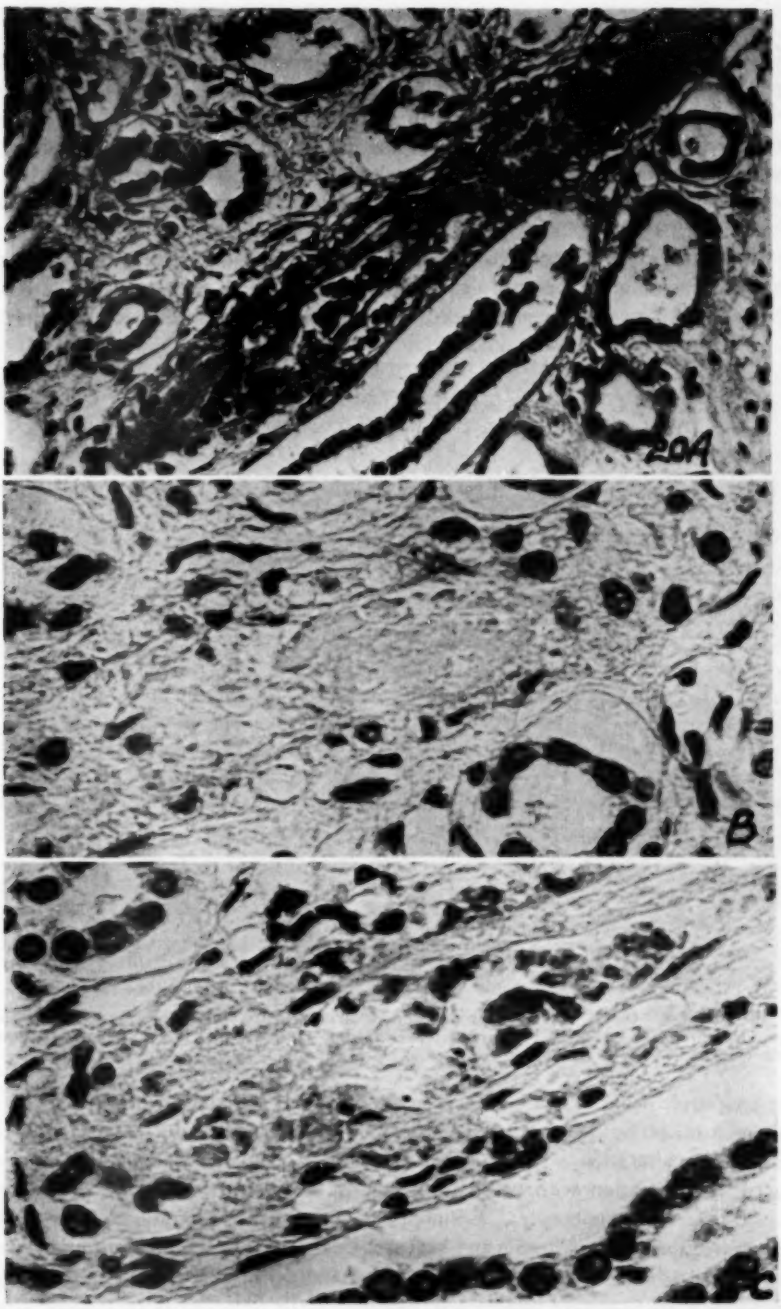

Fig. 20 (Case 14549).—*A*, interlobular artery of kidney. Wratten B filter (green); × 200. Longitudinal section showing at the inferior end a large irregular clump of fibrinoid, homogeneous in appearance with adjacent smaller clumps. Upper end shows several smaller separated clumps of similar appearance. Lumen contains hematoxylin bodies. *B*, lower end of vessel in figure 20*A*. Wratten A filter (red); × 600. Note the almost white appearance of the fibrinoid mass. *C*, upper end and lumen of vessel in figure 20*A*. Wratten A filter (red); × 600. Note the dark-stained intraluminal hematoxylin bodies and the paler but still gray clumps of blue-smudged fibrinoid.

Careful examination of areas in the heart showing fibrinoid connective tissue alteration discloses that the eosinophilia of the fibrinoid material is sometimes dulled by bluish-purple particles embedded in and blending with the acidophilic matrix (Fig. 17*A* and *B*). These particles give the Feulgen reaction (Fig. 18). Such foci are found surrounded by and merging with areas of purely eosinophilic fibrinoid material disposed within more or less heavily inflamed connective tissue septa (Fig. 19).

In arteries displaying conspicuous eosinophilic fibrinoid alteration, hematoxylin-stained elements are often seen distributed in an identical manner as in the heart. They are Feulgen-positive. Advanced vascular lesions appear to have rather faint

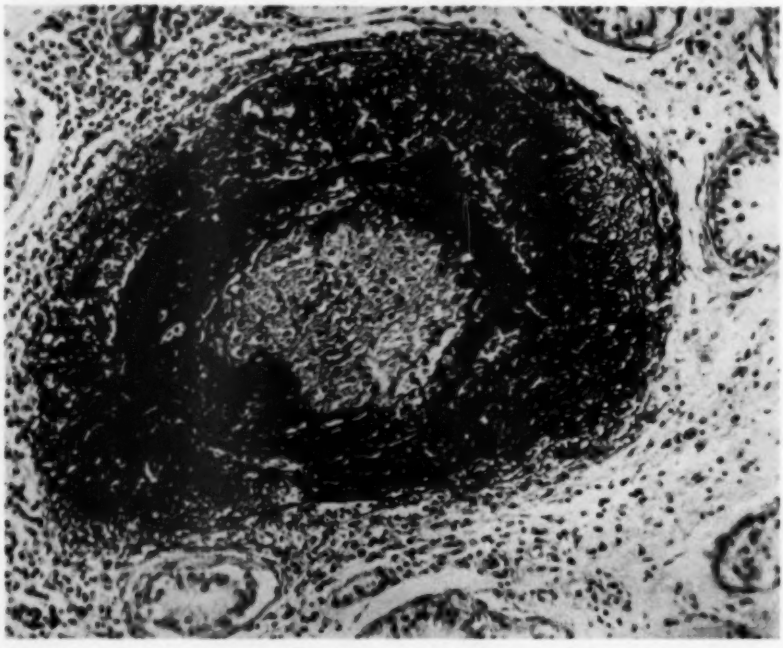

Fig. 21 (Case 14549).—Testis. Phosphotungstic acid hematoxylin (PTAH) stain; × 150; Wratten B filter (green). Artery with advanced fibrinoid necrosis showing bandlike and clumpy homogeneous deposits in the intima and media, with centrifugal radiating fine fibrin fibers.

smudgy purple hematoxylin bodies (Fig. 20*A*, *B*, and *C*) embedded in a smooth or clumpy eosinophilic matrix that tends to blot out the media and extends into the intima. This alteration is often associated with a severe dense inflammation evidenced by infiltration with polymorphonuclear leucocytes, histiocytes, and a few plasma cells and lymphocytes. Eosinophiles are rare. Proliferation of the endothelium is often seen. Pyknosis and karyorrhexis of cells in the area are found, and much nuclear debris is accumulated, deep blue and dense in hematoxylin and eosin stains. In some examples of this arteritis, fine fibrils like true fibrin radiate from the homogeneous media into the adventitia. These fibrinous deposits intensify the homogeneous appearance of the affected vascular wall. In phosphotungstic acid and

Mallory's trichrome stains, however, the inner mantle zone differs from the outer layer because of its coarsely fibrillar and homogeneous clumpy appearance and sometimes lighter color tints (Fig. 21).

In advanced phases the intimal, elastic, muscular, and fibrous layers are fused into a homogeneous, sometimes segmental, mass deforming the wall. Studies of these advanced lesions were undertaken with the idea of finding out whether one tissue component was obliterated or destroyed more than another. Digestion studies with trypsin and with hot 5% trichloracetic acid followed by special stains revealed that the loss of elastic staining appeared to be due partly to a coating of the elastic fibers by an insulating eosinophilic mass, as hot trichloracetic acid treatment uncovered elastic fibers easily stained by Weigert's elastic stain, which in control adjacent

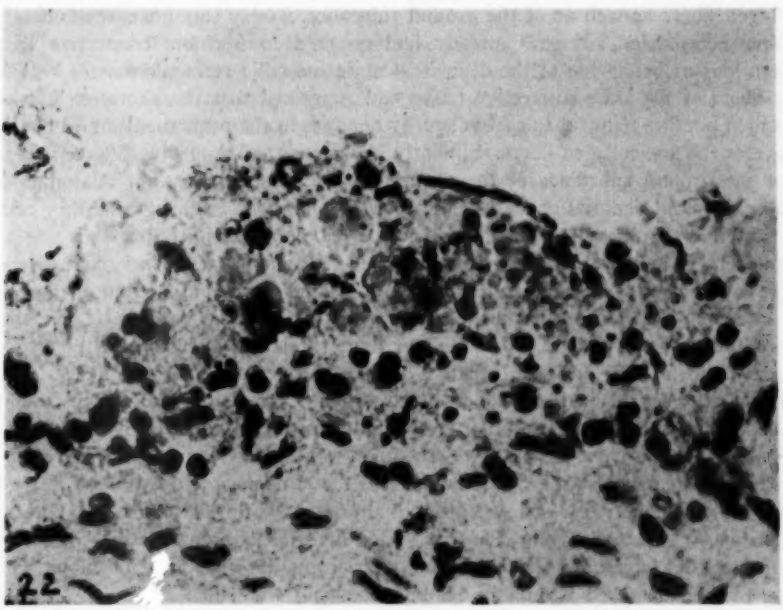

Fig. 22 (Case 14416).—Lung. Pulmonary artery branch. Hematoxylin and eosin; × 600; Wratten A filter (red). Beneath the endothelium there is an accumulation of clumps of varying size showing distinct dark smudging. There is much nuclear debris which is sharply outlined and very dark. Polymorphonuclear leucocytes and lymphocytes infiltrate the inner zone of the media.

undigested sections did not stain. The fate of the muscle fibers could not be traced. Collagen fiber stains showed a moderate residuum of collagen fibers in the middle of these lesions. Toluidin blue stains showed little or no metachromasia in these "fibrinoid" vessel walls.

In contrast to the severe arteritic involvement associated with segmental or circumferential fibrinoid necrosis of the vessel wall, the less advanced lesions show isolated deposits of bluish smudged eosinophilic material in the subendothelial space (Fig. 22) and between the muscle cells of the media. These deposits are associated with little or no evidence of inflammation. Arterial lesions of all grades of severity

can be seen, not only in the same case but even in the same organ. The observations of such early lesions have led us to believe that the fibrinoid vascular changes in systemic lupus erythematosus are the result of an intramural deposition of degraded nucleoprotein from the blood stream.

COMMENT

When Klemperer, Pollack, and Baehr, in 1941,[8] stressed the significance of the systemic fibrinoid connective tissue degeneration, they merely gave a descriptive characterization of a fundamental feature of the morphologic changes in systemic lupus erythematosus. In their interpretation of fibrinoid connective tissue degeneration they modified the concept originally presented by Neumann [25] by maintaining that this alteration denotes a change in the physicochemical constitution of the collagen fibers as well as of the ground substance. Today this interpretation is no longer acceptable. Klinge [26] already had referred to fibrinoid connective tissue alterations as being due to the deposition of eosinophilic refractile masses between the fibers of the loose connective tissue and suggested that the alteration of stout collagen bundles might result from similar deposits in the cement substance binding together the fine collagen fibers. Similarly, Altschuler and Angevine [27] have stressed that the ground substance of the connective tissue is the constant anatomic site where fibrinoid deposits occur. The analysis of the fibrinoid changes as they occur in systemic lupus erythematosus makes it even more obvious that they are not dependent on collagen fiber alteration. In 1941 Klemperer, Pollack, and Baehr [8] were forced to regard the "wire loops" as a fibrinoid alteration of ground substance in order to make this conspicuous lesion conform with their general thesis. Furthermore, the glomerular "hyalin thrombi" were interpreted as intrusions of the swollen basement membrane into the capillary lumina, a concept we hold unlikely. These conclusions were reached because of tinctorial identity of tissue changes but failed to consider the actual material constitution of the substance which is responsible for the altered microscopic appearance. In other words, they limited themselves to a descriptive characterization of the connective tissue changes but did not enter into the question of the nature of the fibrinoid alteration or the existence of a fibrinoid substance.[28] The investigations here reported originated in observations of eosinophilic deposits which, because of their tinctorial quality, resembled fibrinoid. They were first encountered within the lymph nodes and were identified with degraded nucleoprotein. These observations attracted our attention to similar deposits which were encountered in situations that could be regarded as unequivocal evidence of fibrinoid connective tissue damage. These findings permitted an integration of the two hitherto unconnected histologic criteria of the basic tissue changes in systemic lupus erythematosus, the hematoxylin-stained bodies and the generalized fibrinoid connective tissue damage. The presence of hematoxylin-smudged eosinophilic material within vascular lumina suggests that the fibrinoid alteration of blood vessels is the result of a passage of degraded nucleoprotein circulating in the blood stream into the adjacent vascular wall. The deposited abnormal substance provokes a nonspecific inflammation, with exudation of fine fibrils of fibrin, whereby the eosinophilic homogeneous appearance of the implicated vascular wall is accentuated, with the resulting picture of a necrotizing arteritis. Similarly, the deposit of smudged eosinophilic masses within the loose connective tissue of the heart is generally associated with inflammation.

The fibrinoid alterations of the connective tissue, and especially of the blood vessels, have hitherto been regarded as a conspicuous but not characteristic feature of the pathologic-anatomic picture of systemic lupus erythematosus. In other words, fibrinoid degeneration was not an adequate criterion for the differential diagnosis of systemic lupus erythematosus. The observations here recorded permit of a separation of the fibrinoid alterations in systemic lupus from those which occur in other diseases, particularly those associated with necrotizing arteritis. This conclusion is based upon the examination of the fibrinoid changes in such cases and equally upon the fact that free hematoxylin-stained bodies have not been found by us in extensive investigations of control material. The fibrinoid material in systemic lupus is the result of a degradation of nucleoprotein initiated by a peculiar disturbance of desoxyribose nucleic acid metabolism which is characteristic for the pathogenesis of this disease.

It is apparent that the mode of formation of this fibrinoid substance makes it unique. Nevertheless, taken alone, without evidence of the evolutionary stages of nucleoprotein degradation, the final product might not, in itself, be distinguishable from other fibrinoid substances with available histochemical methods. Certain protein digestion and solubility studies point to a possible method of identifying the fibrinoid substances as they occur in other conditions.

SUMMARY

A review of 14 new consecutive cases of systemic lupus erythematosus and of control cases confirms the diagnostic significance of the free hematoxylin bodies for the autopsy diagnosis of the disease.

Extensions of the previous histochemical studies show evidence of a progressive degradation of the desoxyribose nucleic acid of the nucleoprotein of the hematoxylin bodies and ultimate complete disappearance of the nucleic acid molecule.

Histochemical investigations centering upon the protein component of the degraded chromatin indicate that it contains a basic protein of nonhistone type.

Histologically and histochemically the fibrinoid material in systemic lupus includes a protein residue of nuclear origin.

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HEMATOPOIETIC RESPONSES TO PROVOCATIVES

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ONE OF the essential needs in hematological investigation is reliable criteria. They should be based on simple methods and arrived at in a short period of time. Evaluation of the number, the type, and the morphology of the cellular elements in the peripheral blood would satisfy these requirements provided they were reliable. The lability of blood cells, and especially of leucocytes, to a multitude of dissimilar injected materials has become apparent in many studies by different investigators. When a material is injected into an animal or man, the investigator must determine whether the responses are of biological significance. The changes may represent physiological or "nonspecific" variations or mechanical errors. Absence of acceptable criteria has plagued the worker in hematology and prevented the acceptance of investigations which, if true, represent significant contributions to our understanding of the hematopoietic mechanism.

Injection of many different substances has produced a leucopenia which is granulocytic and lymphocytic or predominantly the former. The leucopenia has lasted for minutes to many hours. The decrease in leucocytes has been followed in most instances by leucocytosis which lasted for several hours. The leucocytosis is usually neutrophilic, with lymphocytosis appearing sometime during the period of increase of the white blood cells. Variations in the results have been obtained not only by different but also by the same investigators in their own experiments. The differences consist in the duration and the degree of leucopenia and leucocytosis and in the relative participation of lymphocytes and granulocytes. These variations seem to be related to the nature and the quantity of the injected material. Some workers concentrated on the leucopenic manifestations, others on the leucocytic. In some studies, emphasis was placed on the number and the type of white blood cells in the peripheral circulation. In other experiments, varying degrees of correlation were made between the changes in the peripheral blood and in the various organs. The conclusions reached by the investigators may be summarized into the following broad categories: (1) the action is nonspecific and not significant biologically; (2) the action represents a phase of the hematopoietic mechanism.

There are two other possibilities inherent in the various experiments: *A*. A large number of the different materials produce a nonspecific action which appears first, and then they set into motion a phase of the hematopoietic mechanism. *B*. Some materials are basically specific but they evoke an initial nonspecific action, which in

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its turn sets into motion another phase of the hematopoietic mechanism, with a resultant confusion in the interpretation of results. The purpose of the investigation described here is to evaluate the first of these possibilities.

PREVIOUS INVESTIGATIONS

Guest and his co-workers [1] obtained a bovine plasma fraction which produced a leucopenia within three minutes when injected intravenously into dogs and guinea pigs. The fraction was precipitated at 20 to 29% of ammonium sulfate saturation. The decrease in cells was largely in neutrophiles and lasted for 30 minutes to 4 hours. The leucopenia was followed by a leucocytosis and was associated with a reduction in blood pressure. The workers believed that they had isolated a new plasma factor free of fibrinolysin and labeled it vascularin. They were aware of the occurrence of leucopenia following administration of pyrogens * and substances of large molecular weight, such as glycogen, acacia, and starch,[4] but believed that the properties of vascularin differed from the other substances and concluded that the plasma fraction was biologically significant.

Steinberg and Martin † described a leucocytosis following intravenous injections of whole and of fractions of human and rabbit plasma or of leucocytes into rabbits. The plasma fraction differed in chemical properties from vascularin and was obtained from the opposite range, 62 to 68%, of ammonium sulfate saturation. The workers correlated the changes in the peripheral blood with those in organs, including marrow. They disregarded the initial leucopenia of the first 5 to 45 minutes as nonspecific and postulated the presence of a biologically significant expulsion factor concerned with the delivery of polymorphonuclear leucocytes from the marrow to the circulation to replace those destroyed in the physiological process of aging and disintegration. Presence of the factor in leucocytes suggested to the investigators the white blood cells as one of the sources of the factor.

Menkin [7] produced a leucopenia in dogs for two to three hours after intravenous injection of a fraction representing 33% saturation of ammonium sulfate of an acid inflammatory exudate. The worker believed that tissue cell destruction in an area of inflammation released several factors concerned with the production of leucopenia, leucocytosis, fever, and tissue necrosis.[8]

Kemp, Cartwright, and Wintrobe [9] injected subcutaneously into splenectomized rats with a leucocytosis a suspension of acetone-desiccated beef spleen, liver, thymus, muscle, pancreas, adrenal, lung, brain, testis, and kidney and obtained a 60% decrease of leucocytes. The decrease in cells started immediately and reached its maximum in 18 hours. The reduction of granulocytes appeared to be proportional to the dosage of the suspension to a certain point. Both neutrophiles and lymphocytes were decreased. The reduced number of leucocytes was maintained by multiple injections. Adrenalectomy did not alter appreciably the results. The decrease in leucocytes was not as marked in nonsplenectomized rats. Contrary to the experiences of other investigators,‡ Kemp and his co-workers failed to produce a decrease of

* References 2 and 3.

† References 5 and 6.

‡ References 10-13.

leucocytes by the use of proteins, lipids, and carbohydrates. These investigators, on the basis of further studies, using extracts of inflammatory tissue, subscribed to Menkin's concept of a specific leucopenic factor.

Elvidge [14] injected rabbits intravenously with quartz particles and with india ink and found an immediate leucopenia which was followed by a leucocytosis in 24 hours. The degree of leucopenia appeared to be proportional to the amount of material injected.

Weisberger and his associates § injected rabbits with intact rabbit leucocytes. They also used extracts of intact leucocytes or those disintegrated by supersonic vibration. These workers obtained a leucopenia up to four hours, followed in four to six hours by a leucocytosis. The leucopenia was associated with 20% decrease in neutrophilic polymorphonuclear leucocytes. The leucocytosis was due to an increase of these cells by 90% or more. The investigators concluded that granulocytes contain leucopenia- and leucocytosis-producing substances which may be the same that Menkin described.

Doan and his associates [17] produced a leucopenia and a leucocytosis in rabbits by the intravenous injection of sodium nucleinate. They correlated the changes in the blood with those in the organs. The leucopenia was neutrophilic and lasted for several hours. It was followed by a marked leucocytosis, up to 100,000 cells, over a period of four days. In contrast to Kemp and co-workers,[9] Doan and his associates state that the splenectomized animals did not show a leucopenia but only a leucocytosis. From the charts presented by both groups of investigators, it appears that a primary decrease in the number of leucocytes had occurred in the two sets of experiments. The confusion arises in the use of and the implication given to the term "leucopenia." "Decrease of leucocytes" would be a more appropriate characterization, considering the character of the experiments and the degree of cell reduction.

Doan and his associates concluded that the leucopenia was due to the sequestration of the leucocytes by the spleen and that sodium nucleinate represented a stimulus which called forth granulocytes from the marrow. Weisberger and co-workers (1949) and Steinberg and Martin (1949 and 1950), independently and using different approaches, reached conclusions similar to those of Doan and his associates (1928) who by still another approach arrived at the view that a chemical component, which is released by granulocytes at some phase of disintegration, activates the bone marrow to maintain a physiological balance of white blood cells in the circulation. However, these conclusions must be considered as speculative until the criteria used by the three groups of investigators are found to be reliable.

Many investigators accept the concept of "nonspecificity" of the leucopenia-leucocytosis response to the injection of various substances. The term "nonspecificity" implies a lack of biological significance. The workers offer several explanations. Löwit [18] considered that cytolysis within viscera accounted for the leucopenia and stimulation of the marrow for the leucocytosis. Vejlens [4] observed a reduction of blood flow and adhesion of granulocytes to the vascular intima and ascribed the leucopenia to these changes. Essex and Grana,[12] Bassen and co-workers,[13] Webb,[20] and Lawrence [21] supported this view and regarded the spleen as a negligible factor.

§ References 15 and 16.

The segregation of leucocytes was believed to occur essentially in the lung.‖ Doan and his associates [17] rejected the views that leucopenia was due to a vasomotor phenomenon or a change of blood volume or retention of white blood cells in the lung. They insisted that the spleen was solely responsible for the decrease of leucocytes.

EXPERIMENTAL METHODS AND MATERIALS

An analysis of experiments reported by other workers suggested the desirability of a correlation of the changes in the blood and the organs, including the bone marrow, at frequent intervals and for an extended number of hours following the injection of provocatives. We suggest the term "hematopoietic provocative" for those substances which produce blood cell responses differing from physiological. In the preliminary studies, several different provocatives were used. They included bacteria and their fractions, cell-free products of bacterial growth, fractions of various bovine organs, plasma, and milk. The injections were made into rabbits and were single or multiple, intravenous or subcutaneous, and the animals were with or without spleen or adrenals. On the basis of the information obtained from these experiments and for the purposes of clarity and space, only the report of the work with milk by a single intravenous injection will be presented in detail. The remaining data will be used as background material and will be referred to for comparison and illustration.

The milk was filtered for sterility. Rabbits of 3 to 6 months of age were used. Studies of the peripheral blood were made prior to and at frequent intervals after injection of milk. The animals were killed at frequent intervals either with pentobarbital (Nembutal), which produced instantaneous death, or by a blow on the head. Tissue was taken from the ear, the mesentery and from all the organs, including marrow, and was fixed in 10% formalin. The tissues were sectioned and stained with hematoxylin and eosin. Total and differential counts of the peripheral cells and platelets were done at frequent intervals for several days. Cell counts were made on all tissues and correlated with those of the peripheral blood. Since the leucopenic phase after injection of milk occurred during the first hour, data were divided into two parts. One part encompasses the results of the first hour, and the other includes the remaining period.

RESPONSES TO MILK PROVOCATIVE DURING THE FIRST HOUR

For the study during the first hour 25 male checker rabbits were employed. Total and differential counts of peripheral blood cells were made prior to and at 5- to 15-minute intervals after the injection of milk. Three or more animals were killed at intervals of 5, 15, 30, 45, and 60 minutes after the injection. Multiple sections were prepared of all the organs. Histological studies of the tissues included counts of leucocytes in 20 random fields of each section. The limitation and the experimental error inherent in such counts were considered in the evaluation of the results. To evaluate the role of the adrenals they were removed in eight additional rabbits. Milk was injected 7 days, 14 days, and 18 days after adrenalectomy, and the leucocyte content of the peripheral blood and the tissues was studied. The spleen was removed in six other rabbits, and similar studies were done to determine the role of that organ in the hematopoietic responses to provocatives.

Five Minutes After Injection.—Polymorphonuclear leucocytes were found to adhere to the swollen vascular endothelium of the ear, lungs, and spleen and to a lesser degree in the liver. There was capillary stasis in the organs and tissues. Leucocytes in the lung capillaries were increased by 26%. A somewhat similar increase was found in the liver sinusoids and in the splenic vessels. These changes in cell content and those to be considered later are significant differences as determined by comparison with control animals. The other organs did not show a signifi-

‖ References 10, 22, and 23.

cant increase. The granulocytes of the peripheral blood were decreased to 48% of the normal (Chart 2). Granulocytopenia was associated apparently with the migration of granulocytes to the viscera, margination and adhesion of these cells to the capillary endothelium, and a generalized vascular stasis.

Fifteen Minutes.—Peripheral tissues contained a very large number of polymorphonuclear leucocytes in the capillaries. Many of the leucocytes were in the process of migration into the perivascular areas of the tissue and the lungs. Some of the granulocytes were disintegrating. There was vascular stasis in the viscera and in the tissues. Many polymorphonuclear leucocytes crowded the distended lung capillaries. In the spleen a reversal took place in the location of granulocytes, which were decreased in the vascular channels and increased in the pulp by 50% (Chart 3).

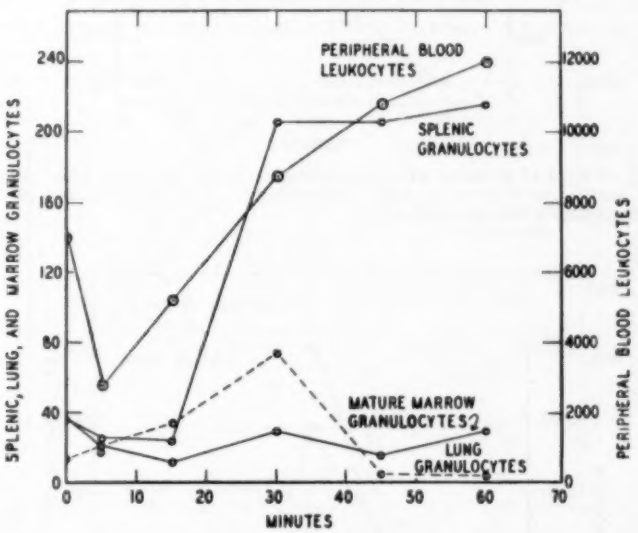

Chart 1.—Counts of leucocytes in the peripheral blood and granulocytes in the lungs, spleen, and bone marrow during the first hour after intravenous injection of milk. The counts represent averages of 25 rabbits. Interpretation: The leucopenic phase in the peripheral blood is followed by a sequestration of granulocytes by the spleen and to a lesser degree by the lungs, the liver, and the tissue capillaries, not indicated on the chart. The marrow granulocytes do not participate significantly in the process at this time.

The organ contained much nuclear debris and many histiocytes with partly disintegrating cells. The liver showed few leucocytes. The bone marrow had a decrease by a third of normal total and mature granulocytes. Leucopenia persisted but to a lesser degree than in the five-minute interval. The significant changes in this period consisted in the perivascular migration of leucocytes, disintegration of the cells, crowding of lung capillaries with granulocytes, and the continued vascular stasis.

Thirty Minutes.—The vascular stasis persisted. Many polymorphonuclear leucocytes were found within and outside of capillary lumina. The leucocytes were in greater numbers in the lungs, spleen, and liver. The bone marrow contained more granulocytes (Chart 1). The circulating leucocytes were increased by one third of

normal (Charts 1 and 2). The increase in peripheral leucocytes was associated with the lowering of the cell content of the marrow in the preceding period (Chart 1).

Forty-Five Minutes.—The vascular stasis persisted. Granulocytes were conspicuously decreased in the lungs, but the general picture was similar to that in the previous period. The total and the mature granulocytes in the bone marrow were

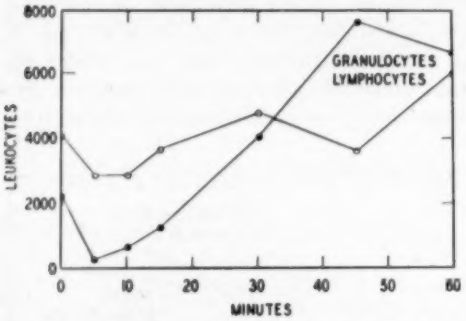

Chart 2.—Counts of granulocytes and lymphocytes of the peripheral blood during the first hour after intravenous injection of milk. Interpretation: The leucopenia and the leucocytosis are determined largely by granulocytes.

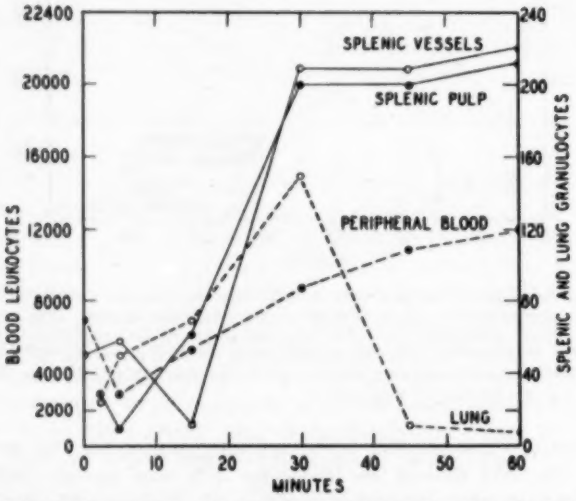

Chart 3.—Counts of granulocytes in the splenic vessels and in the pulp, in the lung, and in the peripheral blood during the first hour after intravenous injection of milk. The counts are averages of 25 rabbits. Interpretation: The chart suggests a rapid migration from the splenic vascular channels into the pulp.

fewer. The circulatory leucocytes were further increased (Charts 1 and 3). The disappearance of leucocytes from the lungs suggests the organ as a passageway. The marrow appeared to have responded to stimuli by the expulsion of polymorphonuclear leucocytes.

One Hour.—Vascular stasis was decreased. Only a few leucocytes were found in the tissues, lungs, and liver. The spleen, however, contained very many leucocytes (Chart 3). The mature granulocytes in the marrow were of normal number, but the immature ones were decreased. It was significant that the leucopenic phase was associated with vascular stasis, adhesion of leucocytes to capillary walls, and vascular flooding of the lungs with granulocytes. The appearance of an extraordinarily large number of granulocytes in the spleen at the termination of the leucopenia suggested the sequestration of those leucocytes which had sustained injury in the vascular stasis and margination. The bone marrow revealed a sensitivity in its responses.

RESPONSES TO HEMATOPOIETIC PROVOCATIVES OVER A PERIOD OF HOURS

For the second phase of the study, 65 rabbits were injected intravenously with milk and killed at hourly or longer intervals. The peripheral blood and the various

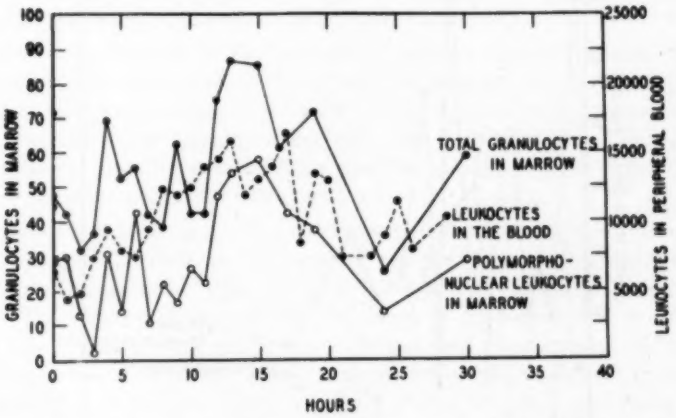

Chart 4.—Average leucocyte counts of the peripheral blood and total and polymorphonuclear granulocytes in the marrow of 65 rabbits injected intravenously with milk. The counts were done for a period of 30 hours after the injection. Interpretation: A leucopenia in the peripheral blood is followed by leucocytosis which fluctuates for a period of 16 or more hours after injection of milk intravenously. The bone marrow appears to supply mature granulocytes to the circulation. The marrow leucocytes multiply in response to the stimulus provided by the provocative. Maturation is increased to replace granulocytes which have been expelled.

organs were studied similarly to the animals in the first phase. When rabbits were injected with milk intravenously, the leucopenia was relatively of short duration. Organ extracts, bacteria, and their chemical fractions induced a leucopenia for 2 to 36 hours. In milk-injected animals the leucocytosis which followed the leucopenia was slight and irregular and was succeeded by a decreased cell content for one to three hours. The secondary leucocytosis began from 7 to 9 hours after injection and in most instances lasted for 12 to 14 hours (Chart 4). The same provocative evoked leucocytosis of varying degrees and periods in different animals. In some instances, leucocytosis persisted for 66 to 72 hours. Some rabbits developed leucopenia but no leucocytosis. These variable results suggest individual variations in the animals. The increase in leucocytes was predominantly granulocytic. Animals

with infections such as pneumonia and with a leucocytosis prior to the injection of milk developed a decrease of leucocytes for a short period but not below the average for a normal rabbit. The leucocytosis in the infected animals was greater than it was prior to the injection of milk. The cell increase lasted for 30 to 48 hours. Although the leucocytosis was largely granulocytic, the lymphocytes also were increased in number.

Two Hours After Injection.—Leucopenia persisted but to a lesser degree. There was a slight vascular stasis. Marrow granulocytes, especially the mature, were decreased (Chart 4). A very large number of polymorphonuclear leucocytes crowded the splenic pulp, and some of the cells were disintegrating (Chart 5). The lung contained an increased number of granulocytes, some of which were disinte-

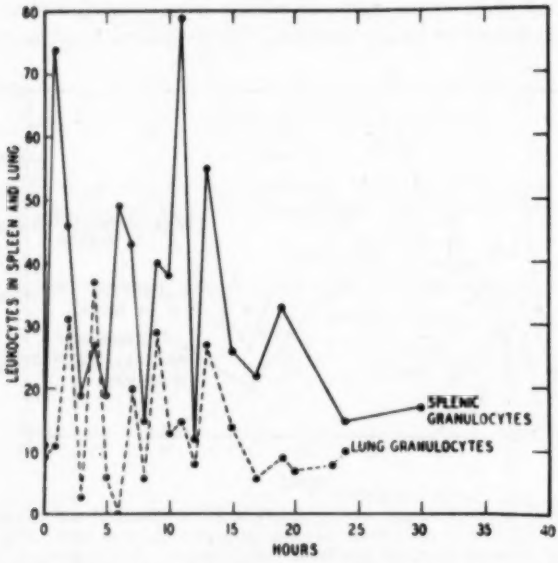

Chart 5.—Average counts of granulocytes in the spleen and lungs of 65 rabbits over a period of 30 hours after injection of milk intravenously. Interpretation: During the leucopenia and leucocytosis the granulocyte content of the spleen and the lungs shows wide fluctuations. Tissue examinations suggest that these fluctuations are due to disintegration of granulocytes in the organs and to redistribution between organs and blood.

grating (Chart 5). It is significant that an expulsion of leucocytes from the bone marrow was not associated with a comparable increase in the peripheral blood. Instead, the cells were found in greater numbers in the spleen and in the lungs. This observation suggests the persistence of the mechanism which excludes granulocytes from the circulation and directs them to the viscera. It is also of interest to note that leucocyte disintegration takes place in the lung as well as in the spleen.

Three Hours.—A slight peripheral leucocytosis which was entirely granulocytic appeared (Chart 4). The marrow became practically depleted of polymorphonuclear cells, with the immature types below normal (Chart 4). The visceral vascular stasis had decreased further. A marked decrease in granulocytes, below normal in the

lungs, was found in the organs except for the liver. Considerable disintegration of leucocytes was present in all the organs. On the basis of these findings, it may be inferred that the circulatory leucocytosis was contributed by the release of the granulocytes from the lungs and the spleen and to some degree from the marrow.

Four Hours.—The peripheral leucocytosis of granulocytes became more pronounced (Chart 4). Vascular stasis had entirely disappeared. All marrow granulocytes returned to a normal number (Chart 5). The adrenals showed presence of foci of polymorphonuclear cells. Leucocyte disintegration became conspicuous in the liver sinusoids. The lungs had acquired once more a large number of granulocytes. The changes observed up to this hour indicated that a maximum period of one hour was required for the marrow to recover from a marked loss of mature granulocytes. There is further indication of the participation of all organs in polymorphonuclear cell destruction. It appears that granulocyte destruction in these periods was achieved essentially by disintegration and only slightly by phagocytosis. The circulating blood participated in the cell destruction (Chart 10).

Five and Six Hours.—In this period there was an attempt for stabilization of the peripheral leucocyte content. The primary leucocytosis, which reached its peak in three to four hours, began to level off (Chart 4). Granulocytes were the cells primarily involved. The lungs were practically free from leucocytes. The number of polymorphonuclear cells fluctuated in the marrow, spleen, and liver (Chart 5), with much cell disintegration in the latter two organs.

Seven to Eighteen Hours.—This was the period of peripheral leucocytosis, which varied in degree and duration. The general pattern, however, remained the same (Chart 4). Lymphocytes were increased in number at occasional and irregular periods. The number of granulocytes fluctuated in the spleen and in the lungs (Chart 5). Disintegrating leucocytes, both free and phagocytosed within large histiocytes, were found in the organs, irrespective of the number of granulocytes they harbored. In the later hours leucocyte destruction by phagocytosis became predominant in the lungs as well as in the spleen. After the 15th hour there was a return of the organs to a normal granulocyte content and leucocyte destruction was no longer apparent in the lungs but persisted in the spleen up to the 30th hour. The number of leucocytes in the marrow also fluctuated. However, a somewhat persistent hyperplasia of mature and immature granulocytes was present during the peak of peripheral leucocytosis (Chart 4). Animals with an infection and a leucocytosis prior to the injection of milk showed a persistence of leucocytosis for 30 to 48 hours. After the 15th hour and with the return of the organs to a normal leucocyte content, the lymphatic apparatus, especially of the spleen, became and remained hyperplastic for one to three hours. A significant relationship appeared in these experiments. There was more than a casual association of leucocyte disintegration on the one hand and the expulsion of granulocytes from the marrow with an increased speed of maturation and a visceral and peripheral leucocytosis on the other hand. The obvious question arose: if disintegrating leucocytes released an active principle which acted on the bone marrow, what mechanism had stopped the otherwise continuous process of leucocyte expulsion and subsequent disintegration? Three possibilities came to mind. The physical changes produced by the

injected foreign substance no longer existed. An antiprinciple developed which neutralized the action. Destruction of granulocytes by phagocytosis instead of disintegration interfered with the formation of an active principle.

INJECTION OF MILK INTO ADRENALECTOMIZED AND SPLENECTOMIZED ANIMALS

In order to evaluate the role played by the adrenals and the spleen, adrenalectomy was performed on eight and splenectomy on six rabbits. Sham adrenalectomy was performed on six other rabbits. All these animals were subjected to procedures similar to those described in previous experiments. Injections of cortisone and

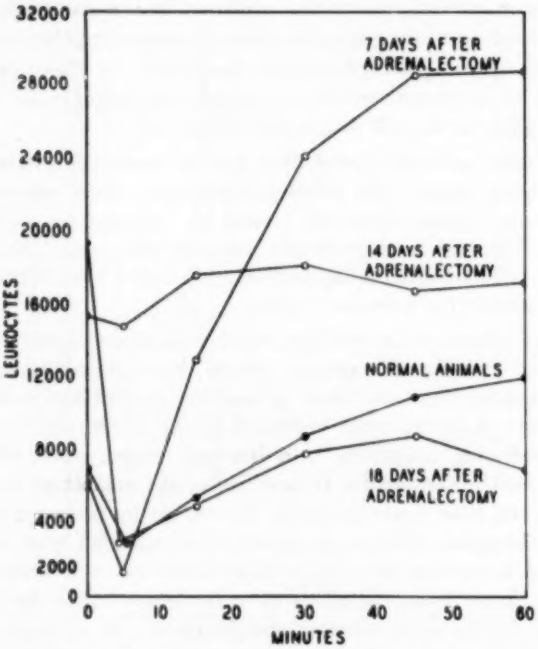

Chart 6.—Average leucocyte counts in six bilaterally adrenalectomized rabbits in the first hour after intravenous administration of milk. Milk was injected at various intervals after adrenalectomy. Interpretation: Adrenals appear to stabilize but not alter basically the leucocytic response to the injection of milk. When the remaining adrenal tissue takes over, as determined by requirement for NaCl, the leukocytic response approaches that of normal animals.

corticotropin into other animals did not contribute materially to the subject and hence will not be described. Bilateral adrenalectomy in rabbits resulted in a marked reduction but not in complete suppression of adrenal activity. These animals have other adrenal tissue distributed in various sites inaccessible to removal. The adrenalectomized animals were maintained on 0.9% sodium chloride in drinking water. For the purposes of these experiments, bilateral adrenalectomy was adequate.

Milk was injected 7, 14, and 18 days after adrenalectomy. The general pattern of response in the peripheral circulation and in the organs was similar to that of

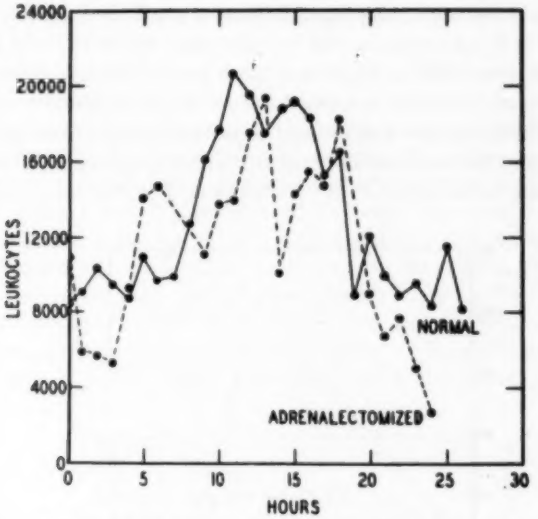

Chart 7.—Average counts of peripheral blood leucocytes in six rabbits with both adrenals removed. Counts were taken for 24 hours after intravenous injection of milk. Comparisons with average counts in normal animals injected with milk are indicated. Interpretation: The adrenals do not exert any basic influence on the leucocyte response-pattern after injection of milk.

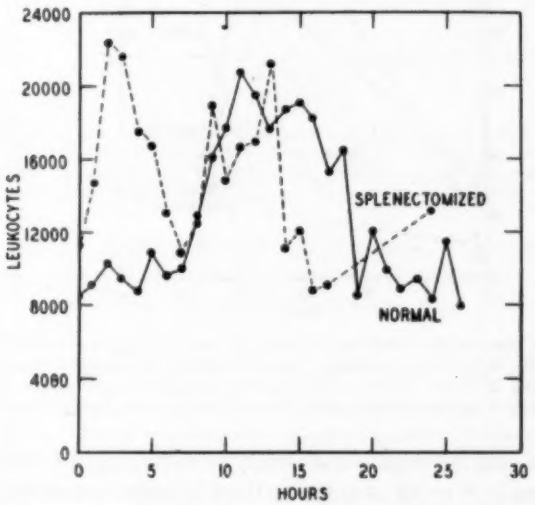

Chart 8.—Average counts of peripheral blood leucocytes for a period of 24 hours in six splenectomized and six normal rabbits injected intravenously with milk. Interpretation: Leucocytosis in the early period after injection of milk suggests that the spleen is a significant organ in the sequestration of leucocytes in that period.

animals with intact adrenals. The differences appeared in the more exaggerated responses as seen in the more pronounced fluctuations (Chart 6 and 7). Leucopenia was participated in by granulocytes and lymphocytes. However, only granulocytes contributed to the leucocytosis. In 18 days after adrenalectomy, when presumably the remaining adrenal tissue had assumed a greater degree of function, the responses after injection of milk simulated those seen in nonadrenalectomized animals. These experiments suggest that the adrenals serve as stabilizing influences of hematopoietic activity in response to the injection of provocatives. The literature on the effect of

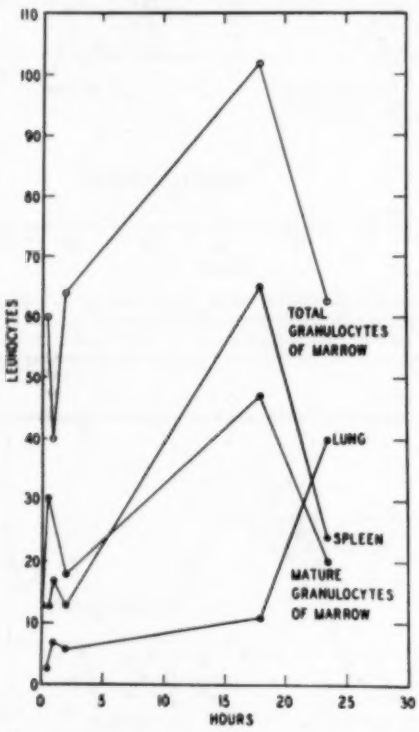

Chart 9.—Average granulocyte counts for 24 hours of marrow, lung, and spleen in a group of 12 rabbits injected with milk subcutaneously. Interpretation: The pattern of response of the bone marrow, lungs, and spleen is similar to that observed in animals injected intravenously. The differences are in degree of response and in time of appearance of the changes.

adrenals upon blood cells is extensive and sometimes disturbing. Effects of injection of a substance acting as a provocative are ascribed by some writers to the adrenal cortex. Insufficient testing of the blood cells interfered with the evaluation of some experiments. Lewis and Page [25] injected typhoid vaccine intraperitoneally into adrenalectomized rats and noted in two hours a decrease of lymphocytes and an increase in polymorphonuclear neutrophiles. They concluded that the adrenal cortex hormones augmented the number of granulocytes in their response to typhoid

vaccine. The general impression gained from the various studies ¶ gives an implication of a modifying influence exerted by the adrenal cortex on the hematopoietic system.

Milk was injected 7 to 14 days after splenectomy. Leucopenia appeared in rabbits at the same time and in the same degree as in the normal and sham-operated animals. However, the recovery from the leucopenia in the splenectomized animals occurred sooner. In 30 minutes after the injection the splenectomized animals had a significant leucocytosis, which increased and reached its peak in two hours (Chart 8). These findings add further data to the view expressed previously that the primary leucopenia is determined by vascular stasis and the accompanying changes. Persistence of the leucopenia is due, apparently, to the sequestration of granulocytes by the various organs and especially the spleen (Chart 8). The general pattern of leucocyte response in the circulation and in the viscera remained the same as in nonsplenectomized animals (Chart 8). That the spleen represents a significant reservoir and an organ which sequesters and redistributes leucocytes may be

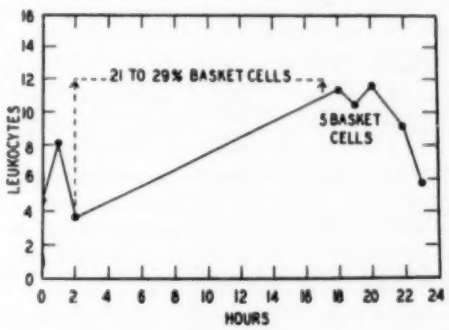

Chart 10.—Average leucocyte counts of peripheral blood in six rabbits injected intravenously with milk. The number of basket cells was counted and correlated to the total leucocyte count. Interpretation: Following intravenous injection of milk, granulocyte destruction in the blood is increased appreciably. The blood participates in leucocyte destruction.

deduced from the larger than usual accumulations of granulocytes found in the lungs, liver, kidneys, heart muscle, and tissues in the splenectomized animals. It appeared that the spleen exerts a stabilizing effect upon hematopoietic responses to provocatives.

INJECTION OF PROVOCATIVES SUBCUTANEOUSLY

Subcutaneous injection of milk was done on 36 rabbits. Blood and tissue studies were similar to those of intravenously prepared animals. A group of 36 rabbits which were injected intravenously or subcutaneously with extracts of bone marrow were used as controls for comparison. Leucopenia in animals injected with milk subcutaneously appeared in two to three hours and lasted for a maximum of three hours. The degree of leucopenia and leucocytosis was less pronounced than in the intravenously injected animals (Table). Chemical fractions of bone marrow which have been tested in this laboratory for biological activity showed a similar pattern of

¶ References 26-32.

variations in animals injected by the two routes (Table). Whether the prolonged duration of the leucopenia with bone marrow extracts is of biological significance remains to be determined. Inflammation of the subcutaneous tissue was found in many of the animals but not in all of those given subcutaneous injections, either of milk or bone marrow. The tissue changes showed a pattern which was similar to that observed with provocatives given intravenously (Chart 9). The responses were delayed and occasionally were more pronounced, but the pattern remained the same.

COMMENT

The experiments outlined in this investigation suggest strongly that the leucopenic phase is produced, at least initially, by vascular stasis and resultant migration and injury to leucocytes. Sequestration by the spleen of the injured granulocytes

*Differences in Responses of Leucocytes in Peripheral Blood Between Animals Injected with Provocatives Intravenously and Subcutaneously**

Provocative	Injection Route	Leucocyte Count Before Injection			Onset of Leucopenia After Injection	Leucopenia, Duration	Leucocyte Count After Injection of Provocatives		
		Total	Granulocytes	Lymphocytes			Leucopenia, Leucocytes/ Cu. Mm. Blood	Granulocytopenia, Granulocytes/ Cu. Mm. Blood	Leucocytosis, Leucocytes/ Cu. Mm. Blood
Fraction "A" of Marrow	Subc.	7,200	2,774	4,526	Between 2 and 3 hr.	66 hr.	1,500-3,800	598-2,280	11,800
Fraction "A" of Marrow	I. V.	7,200	3,168	4,032	Within 10 min.	9 hr.	1,820-2,360	352-728	10,250
Fraction "B" of Marrow	Subc.	9,100	4,280	4,820	After the 3rd hr.	96 hr.	1,800-5,000	1,008-1,500	14,200
Fraction "B" of Marrow	I. V.	7,000	3,010	3,990	Between 5 and 10 min.	9 hr.	1,440-2,660	196-1,040	9,600
Milk	Subc.	9,200	3,680	5,520	Between 2d and 3d hr.	2 to 3 hr.	3,660-4,200	1,044-1,020	14,650
Milk	I. V.	7,000	3,010	3,990	Within 5 min.	Maximum of 1 hr.	1,132-2,510	168-775	18,100

* Interpretation: Leucocyte responses in the peripheral blood are delayed after subcutaneous injections of provocatives, but the duration of the response is longer than that following intravenous injections. The general pattern of response is similar in both routes.

and their disintegration in that organ and in the lungs and the liver, as well as in the circulation, serve to maintain the leucopenic state. The results of our investigation are not in agreement with the contentions of investigators who believe that one organ such as the lungs or spleen or one process such as intravascular cytolysis, vascular stasis, or redistribution of leucocytes is responsible for the leucopenia. Since the leucopenia and the visceral changes are essentially similar no matter which provocative is used, it may be concluded that leucopenia is a nonspecific response. This response in our experiments appeared to be modified but not altered basically by the adrenals and the spleen.

The phase of leucocytosis appeared to be contingent upon the disintegration of granulocytes in the circulation and in the various viscera. There is no indication that the lung exercised any peculiar function in removing leucocytes except as a passage-way and by cell disintegration. The latter process is shared by the liver, lymph nodes, blood, and, to a greater degree, by the spleen. The experiments recorded in this investigation suggest that leucocytosis which occurred after injection

of a provocative was a specific phenomenon and a part of the hematopoietic mechanism which regulates the number of leucocytes in the circulation and in the bone marrow. These experiments are interpreted by us to indicate that a nonspecific leucopenia, which may be produced by many different substances, gives rise to a release of some substance in the disintegrating leucocytes. This substance, labeled by us "expulsion factor," # sets into motion a phase of a specific hematopoietic mechanism which mobilizes mature leucocytes from visceral depots, releases mature granulocytes from the bone marrow, and stimulates maturation of immature granulocytes. It appears that the adrenals and the spleen stabilize the leucocytosis as well as the leucopenia. This investigation suggests a relationship to the responses seen in an inflammatory process. Inflammation is attended by a pattern of variable leucopenia and leucocytosis.[24] An area of inflammation gives rise to substances which include disintegrating leucocytes, tissue debris, and bacteria and their products. Repeated absorption of these noxious substances may be considered as provocatives which produce vascular stasis and migration and disintegrations of leucocytes. Duthie and Chain [33] and Moon and Tershakovec [34] are essentially in agreement with this concept. They found that a number of different substances derived from the tissues, and not a single specific substance, initiate an inflammatory reaction. Our experiments do not indicate the presence of a leucopenic factor in the leucocytes, as suggested by Weisberger and co-workers.*

The events which follow the injection of a provocative represent a sequence which may be summarized as follows: Upon injection of a provocative, a leucopenia appears within a few minutes and lasts for minutes to several hours when the intravenous route is used. On subcutaneous injection, with or without a resultant inflammation, the leucopenia is prolonged. Some provocatives such as organ extracts produce a leucopenia which lasts for three to five days. A question may be raised whether these extracts are biologically significant. The leucopenia obtained by provocatives is associated with a diffuse vascular stasis; a redistribution of granulocytes in the blood, lungs, liver, and spleen; adhesion of granulocytes to the endothelium, and their passage through vascular walls into the surrounding tissue. The granulocytes appear to become damaged in these processes and disintegrate intravascularly and in the lungs, liver, and spleen. This granulocytic disintegration appears to be associated with peripheral leucocytosis. The peripheral leucocytosis is associated with a reduction of mature granulocytes in the marrow, suggesting the marrow as the source of leucocytes. The increase of granulocytes in the circulation fluctuates with that in the marrow and in other viscera. The decline in the number of granulocytes in the liver, lungs, and spleen is associated with cell disintegration and phagocytosis.

SUMMARY

Milk and other substances were injected into rabbits. The changes and leucocyte content in the peripheral blood and in organs were studied and correlated. The term hematopoietic provocative is applied to these substances. These investigations suggest that the leucopenia is nonspecific. The subsequent leucocytosis, however, represents a phase of the hematopoietic mechanism, probably produced by some substance released during the process of granulocyte disintegration.

References 5 and 6.

* References 15 and 16.

The leucopenia is due initially to vascular stasis, adhesion of granulocytes to the vascular endothelium, and redistribution of leucocytes to various viscera.

Following the initial phase of leucopenia, granulocytes migrate from the vascular lumina and disintegrate. This disintegration takes place also in the lungs, liver, and spleen and in the circulation. No single organ is responsible for leucocyte destruction.

The spleen and the adrenals appear to stabilize the responses of leucocytes but do not change the basic pattern of the response.

The bone marrow responds to the stimulus supplied probably by disintegrating leucocytes. Granulocytic hypoplasia and hyperplasia follow the injection of provocatives.

The various changes which are described in the peripheral circulation and in the organs represent a pattern. Knowledge of this pattern may aid in differentiating nonspecific from biologically significant responses. On the basis of these experiments, it seems unlikely that a single injection of a substance represents a satisfactory procedure for determining the effect upon the hematopoietic mechanism.

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PATHOLOGY AND PATHOGENESIS OF SPERMINE-INDUCED RENAL DISEASE

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SPERMINE is an aliphatic amine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$] that is widely distributed in mammalian tissues. The highest concentration of this substance is in the prostate gland.* Generally, it has been considered along with the related amines putrescine, cadaverine, and spermidine to be of low toxicity, with action upon the central nervous system. Little is known concerning the physiologic significance of spermine, although in vitro it has been found to inhibit the oxidation of carbohydrate in brain tissue,[4] to antagonize the bacteriostatic action of atabrine on Escherichia coli,[5] and to possess under certain circumstances a bacteriostatic action on the tubercle bacillus.[6]

Contrary to previous concepts ascribing a neurodepressant action to spermine [2] and the related amines, we have observed that spermine produces a severe and frequently fatal renal lesion in the small animals tested. No lesions of the central nervous system have been evident. In addition, spermidine, putrescine, and cadaverine have not been toxic. Certain diamines, ethylene diamine and trimethylene diamine, however, are capable of producing a similar lesion. This is also true for bromethylamine, but diamines of a chain length greater than three carbon atoms and other monoamines were ineffective in producing pathologic change.

The purpose of this communication is to describe the pathologic features of spermine-induced renal disease and to comment on the pathogenesis of the lesion produced.

METHOD AND MATERIALS

The spermine utilized in this study was three commercial samples of spermine 4 HCl.† One was recrystallized as the phosphate, and another was put through one recrystallization as the hydrochloride and two as the phosphate. A further sample of spermine was obtained as the phosphate from the National Institute for Medical Research of Great Britain. Since all the samples demonstrated equivalent activity in so far as producing renal lesions they shall be referred to hereafter as spermine. A sample of spermidine was obtained from Dr. H. P. Schultz,‡ while putrescine and cadaverine were obtained from commercial sources, and doses of 0.5% solutions in 0.9% saline were employed.

The animals used may be divided into several study groups.

From the Laboratory of Pharmacology and Pathology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

*References 1, 2, and 3.

† From Hoffmann-La Roche, Inc., Nutley, N. J.

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I. Pathologic Effect of Spermine.—There were 49 mice in this group, all of the National Institutes of Health general purpose stock, upon which a morphologic study of the effect of spermine administration was undertaken. There were 4 males and 45 females. Spermine was administered by the intraperitoneal route in 42, by subcutaneous injection in 3, intravenously in 2, and orally in the diet of 2. In addition four rats (200 gm.) and two small rabbits (1,200 gm.) were also given spermine intraperitoneally. The dosage schedule and periods of observation are listed in Table 1.

II. Pathologic Effect of Putrescine, Cadaverine, and Spermidine.—A total of 12 general purpose stock mice were given intraperitoneal injections of these related amines daily for five days. Five received putrescine, five cadaverine, and two spermidine.

III. The Pathologic Effect of Other Amines.—Four mice of the general purpose stock received trimethylene diamine intraperitoneally (300 mg. per kilogram, one injection) and were killed one, two, three, and four days after the injection. Four mice were given one intraperitoneal injection of ethylene diamine (400 mg. per kilogram) and were killed at one, two, and three days after injection. Allyl amine (75 mg. per kilogram, one injection) was administered to two

TABLE 1.—*Dosage and Periods of Observation*

Dose, Mg./K. × Frequency	Mice, No.	Time Killed or Dead, Days
60 × 1	3	1
60 × 1	2	1½
60 × 1 60 × 2	6	2
60 × 1 60 × 3	6	3
60 × 1 30 × 4	7	4
40 × 1 15 × 5	3	5
15 × 6	2	6
60 × 1 15 × 9	2	10
15 × 9	1	21
7.5 × 10 10 × 14	1	28
7.5 × 10 10 × 14	2	30
7.5 × 10 10 × 10 15 × 12	2	35
7.5 × 10 10 × 10 15 × 12	2	36
7.5 × 10 10 × 10 15 × 12	2	38
0.1% in diet	2	70
7.5 × 10 10 × 11	2	73
15 × 7	2	75
20 × 35 30 × 14	2	77
Rabbits, No.		
33 × 1	2	5
Rats, No.		
33 × 1	2	5
33 × 1	1	6
33 × 1	1	7

mice, and these were killed in one and three days. Three mice received a single intraperitoneal injection of bromethylamine (500 mg. per kilogram) and were killed in one, two, and three days. One mouse received one intraperitoneal injection of ethanolamine (1,000 mg. per kilogram) and was killed in four days.

IV. The Effect of Ureteral Ligation on the Production of Spermine-Induced Renal Disease.—A total of eight mice of the National Institutes of Health general-purpose stock and 14 rats of the OM strain were subjected to either bilateral or unilateral ureteral ligation. The procedure consisted of placing a double ligature of black silk about the midportion of either both ureters or one (right) ureter under ether anesthesia. Bilateral ligation was performed on three mice, and spermine (50 mg. per kilogram) was administered intraperitoneally to two of these 24 hours after ligation. One received no spermine and served as a control. All three were killed in 24 hours. Unilateral ligation was performed on the remaining five mice, and spermine (50 mg. per kilogram) was injected intraperitoneally 24 hours after ligation. Two of these five were killed in 24 hours and the remaining three in 48 hours. Two of the 14 rats had bilateral ureteral ligation performed and received an intraperitoneal injection of spermine (40 mg. per kilogram) 24 hours after ligation. They were killed 24 hours after the injection. Three underwent unilateral ligation and were given spermine 40 mg. per kilogram) intraperitoneally 48 hours after the operation and were killed 48 hours later. Another three that had unilateral ligation were given an intraperitoneal injection of spermine (40 mg. per kilogram) 24 hours after ligation and were

killed in another 24 hours. One rat was given spermine (40 mg. per kilogram) seven days after unilateral ligation and was killed 24 hours later. Three rats were subjected to unilateral ureteral ligation for one, two, and seven days but did not receive spermine and acted as controls. Two additional rats underwent ureteral ligation (one unilateral, the other bilateral) and after 24 hours received 2 L. D. of bichloride of mercury and were killed 24 hours after the intraperitoneal injection.

Reparation of Specimens.—Kidney, adrenal, liver, and spleen from all animals as well as the brain, spinal cord, lungs, heart, gastrointestinal tract, and urinary bladder of many were fixed in 10% formalin. In some instances these tissues were fixed in Zenker acetic fluid. Many kidneys were also fixed in 65% alcohol for alkaline phosphatase study according to the method of Mowry.[7] Except for this latter procedure, sections were taken to water in the usual manner. All staining methods employed were according to the techniques described by Lillie.[8]

Routine Stains on All Tissues:

1. Hematoxylin and eosin
2. Periodic acid-Schiff method, alum hematoxylin counterstain

Other Procedures:

1. Alkaline phosphatase method on kidney sections
2. von Kossa procedure for mineral salts
3. Oil red O method for lipids performed on formalin-fixed frozen sections
4. Feulgen nucleal procedure on kidney sections
5. Thionin, pH 4 (1:10,000), for one-half hour after digestion of kidney sections with crystalline ribonuclease 1:10,000 (Armour Lot 7095X) in pH 6 M/phosphate buffer for one hour at 37 C.; control sections of rat pancreas similarly treated
6. Ferrocyanide reaction (Perls) for iron
7. Digestion of sections of liver with barley malt diastase, 0.1% in saline phosphate buffer pH 6 for one hour at 37 C., followed by the periodic acid-Schiff method; these were compared with duplicate untreated sections; enzyme activity control consisted of heavily glycogenated liver sections
8. Observation for birefringent particles with the polarizing microscope on fresh and formalin-fixed tissues
9. Sections of kidney, 5 μ in thickness, stained by the periodic acid-Schiff method, were projected on drawing paper with a Scopicon microprojector, and the basement membranes of the glomerular capillaries were traced and their thickness measured; an average measurement of many glomeruli measured in this manner was compared with a measurement obtained from sections of normal kidneys prepared at the same thickness and similarly stained; this offered an objective method for the determination of basement membrane thickness.

RESULTS

Pathologic Lesion Produced by Spermine.—A characteristic renal lesion was produced in mice (male and female), rats, and rabbits by the administration of spermine by either the intravenous, subcutaneous, or intraperitoneal routes. Spermine incorporated into the diets (0.1%) of several mice for 70 days failed to produce morphologic change. Grossly, the kidneys after parenteral spermine administration appeared swollen. The capsules were thin and not adherent to the cortices, which were cream-white. The medullas were congested. Microscopically, the lesion may be classified as a proximal nephron nephrosis. Early (one day) changes consisted of hyaline droplet degeneration of many of the epithelial cells of the proximal convoluted tubules, especially in their distal portion or in the outer medullary zone (Fig. 1). In addition, slight fatty change was also observed in many of these cells. These changes were quite diffuse, with only an occasional interposed normal tubule being present. The periodic acid-Schiff stain revealed absence of some of the brush

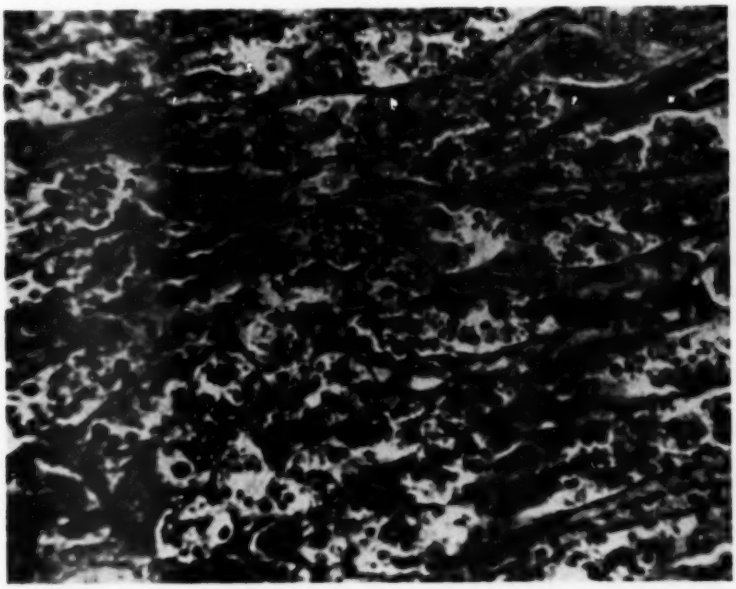

Fig. 1.—Severe hyaline droplet change in epithelial cells of proximal convoluted tubules; mouse; × 345.

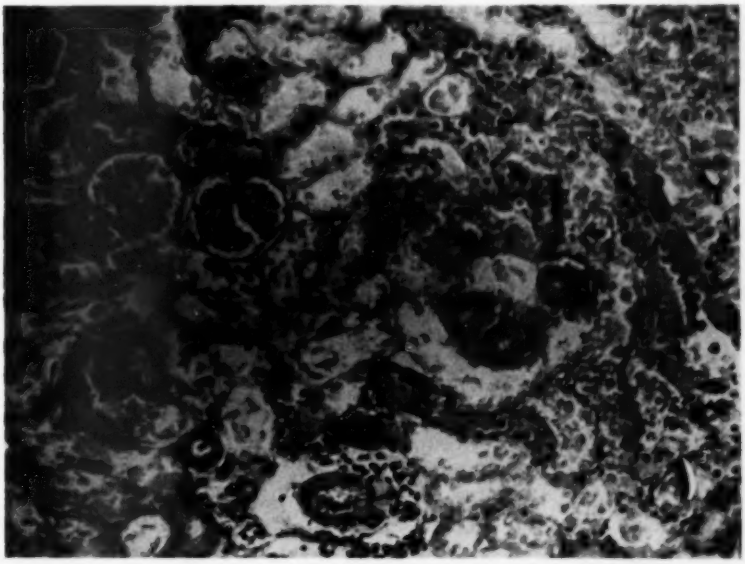

Fig. 2.—Desquamative necrosis of epithelial cells of proximal convoluted tubules; mouse; × 210.

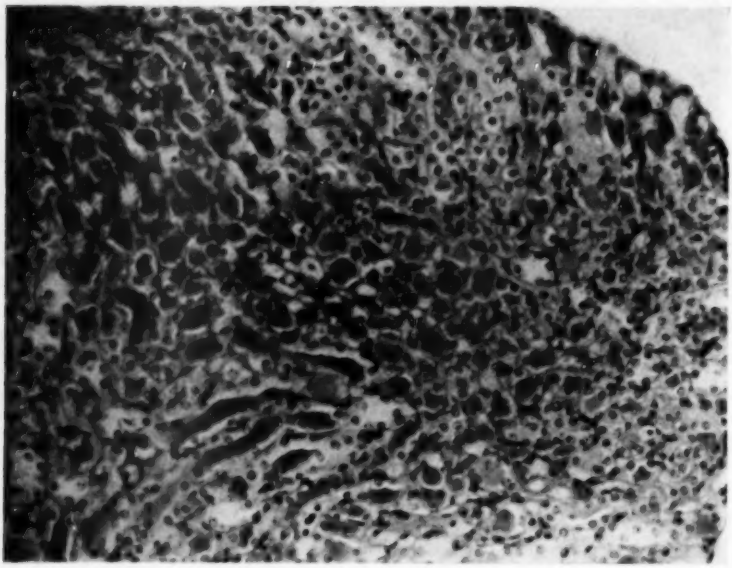

Fig. 3.—"Hyalinoid casts" in collecting tubules; mouse; × 210.

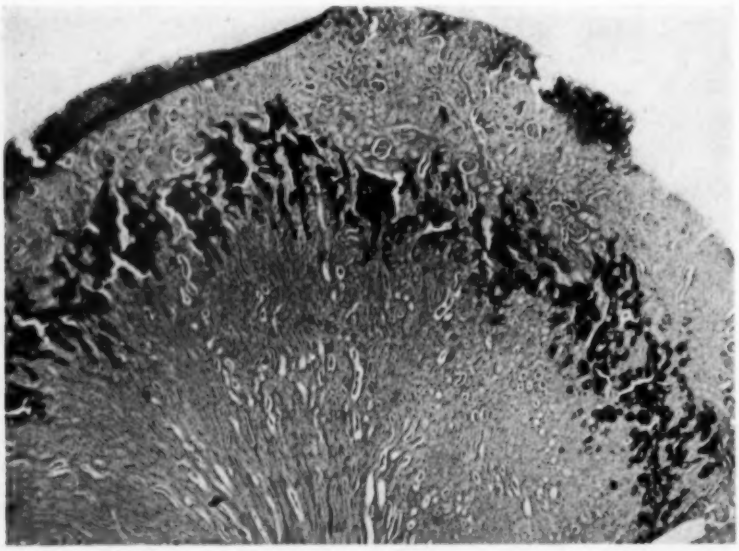

Fig. 4.—Section stained by the von Kossa method, demonstrating mineral deposits in the distal portions of the proximal convoluted tubules (outer medullary zone) only; mouse; × 30.

borders at this time, and alkaline phosphatase preparations at this time demonstrated only slight reduction in activity. The lesion progressed rapidly, for in one and one-half to three days after spermine administration there was a desquamative necrosis of the epithelial cells unaccompanied by reactive changes not only in the distal portion but also in proximal segments of the proximal convoluted tubules (Fig. 2). Numerous casts were evident in the lumina of the collecting tubules (Fig. 3). Many of these were hyalinoid and stained intensely positive with the periodic acid-Schiff method. In addition, many casts contained bluish (hematoxylin and eosin) granular and amorphous material, which demonstrated positive von Kossa as well as Feulgen nucleal reactions but were not colored by the ferrocyanide method for iron or affected by ribonuclease digestion. No birefringent particles were observed. From the results of these methods it appears that the casts consist of protein, mineral (most likely calcium phosphate or carbonate), and nuclear debris. Occasionally at this time, but more frequently at about 10 days after spermine administration, lakes of mineral were noted to occupy entire tubular lumina as well as to replace many of the lining cells of the affected tubules (Figs. 4 and 5). Alkaline phosphatase activity was moderately reduced and was noted to be more diffusely distributed throughout the cells of the proximal convoluted tubules as compared to the distinct ring-like zone of activity in normal controls prepared simultaneously.

Attempts to produce subacute and chronic renal lesions by the daily administration of spermine for varying periods of time were undertaken. After three to five weeks either the acute spermine lesion as described above or no morphologic change was observed in the kidneys. On occasion, evidences of tubular regeneration, consisting of flattened epithelial cells with increased cytoplasmic basophilia, mitotic figures, and depressed alkaline phosphatase activity, were noted. More prolonged administration, as for 8 to 10 weeks, however, quite consistently produced perivascular stromal lymphorrhages (Fig. 6). There were no tubular or other changes evident, except for the presence of regenerative changes on occasion.

At no time were glomerular changes recognized. The thickness of the basement membranes of the glomerular capillaries was not greater than that of normal control animals, when objectively measured. Except for the lymphorrhages about stromal vessels observed after prolonged spermine administration, the stroma and renal vessels were without changes. The juxtoglomerular apparatus or macula densa failed to reveal any deviation from normal.

The livers of four animals that received one injection of spermine and were killed four days later revealed focal necrosis. Similar changes were not observed in the other animals studied. There was no detectable reduction in hepatic glycogen following spermine administration. Other tissues failed to reveal pathologic change.

The Effect of Spermidine, Putrescine, and Cadaverine.—No morphologic changes were observed following the administration of these related amines.

The Effect of Other Amines.—Bromethylamine administration in doses of 200 mg. per kilogram produced a renal lesion similar to that observed with spermine, characterized by a desquamative necrosis of tubular epithelium as well as the deposition of mineral after two days, although death from renal insufficiency did not occur. Ethylene diamine and trimethylene diamine appeared to be less potent, since only moderate tubular damage was evident after four days and this was limited almost exclusively to those segments of the proximal convoluted tubule in the outer

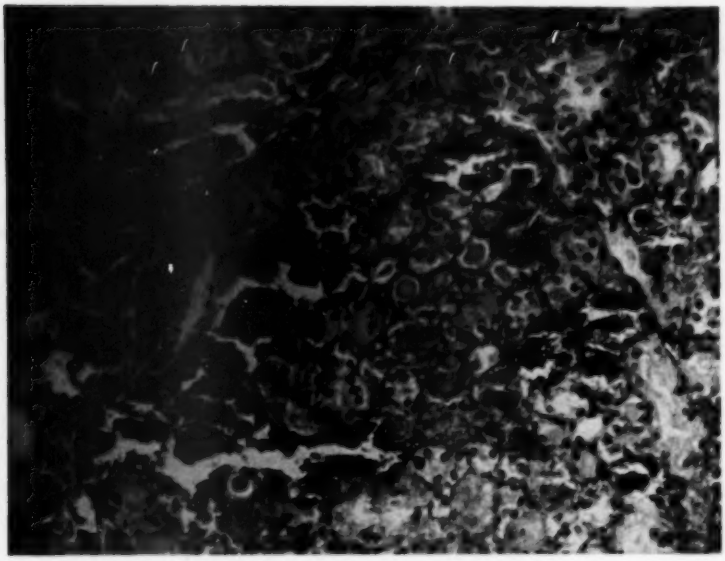

Fig. 5.—Section demonstrating mineral deposits within the lumen as well as replacing epithelial cells of the proximal convoluted tubules; mouse; × 210.

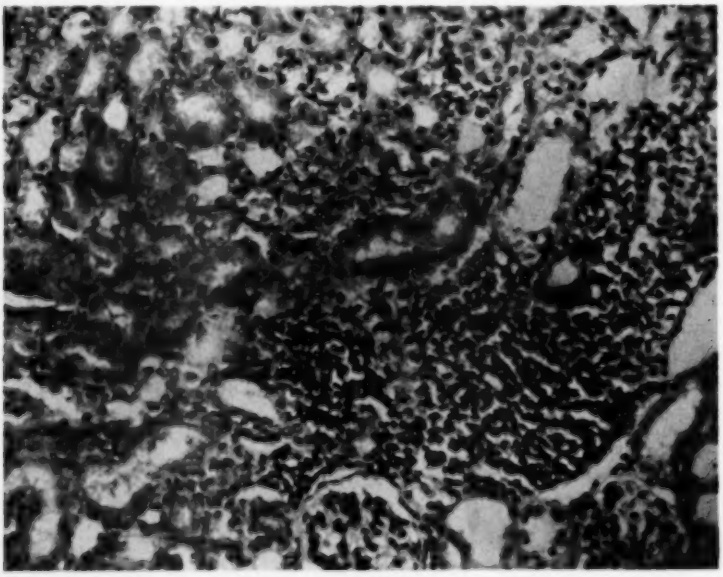

Fig. 6.—Perivascular lymphorrhage after prolonged spermine administration; mouse; × 210.

medullary zone. This resembled the very early (one day) change following spermine administration. All other monoamines were ineffective in producing morphologic change.

The Effect of Ureteral Ligation on Spermine-Induced Renal Disease.—Ligation of either one or both ureters produced hydronephrosis within 24 hours. Microscopically, control kidneys with hydronephrosis revealed only tubular dilitation.

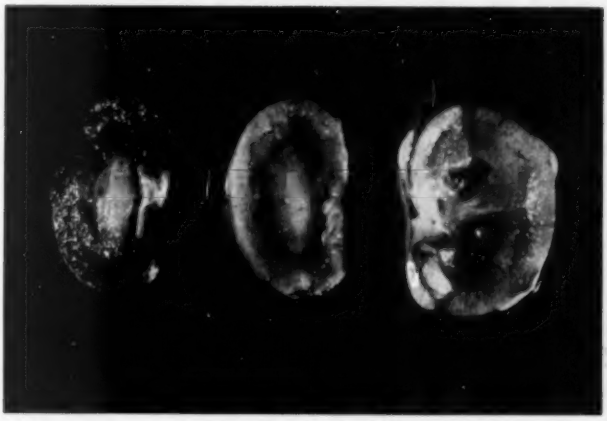

Fig. 7.—Rat kidneys, demonstrating normal control on the left. Compare with nonligated kidney in the center and hydronephrotic kidney on the right, removed two days after spermine administration. Note spermine effect in the latter two.

TABLE 2.—*Results of Ureteral Ligation*

Animals, No.	Ligation Procedure	Total Ligation Time, Days	Time After Spermine, Days	Extent of Lesion	
				Ligated Side	Control Side
Mice					
2	Bilateral	2	1	+++	.....
2	Unilateral	2	2	+++	+++
3	Unilateral	3	2	+++	++++
1	Bilateral	2	0	Control	.....
Rats					
2	Bilateral	2	1	++	.....
3	Unilateral	4	2	+++	+++
3	Unilateral	2	1	+++	+++
1	Unilateral	8	1	++	+++
3	Unilateral	1, 2, 3	0	Controls	.....
1	Bilateral	2	1 ($HgCl_2$)	—	.....
1	Unilateral	2	1 ($HgCl_2$)	—	++++

The administration of spermine subsequent to ligation produced the typical gross (Fig. 7) and microscopic lesion of spermine in both the hydronephrotic and control kidneys as well as in both hydronephrotic kidneys following bilateral ureteral ligation (Table 2). As a control of the method, it was noted that ureteral ligation for similar periods of time was capable of protecting the obstructed kidney from bichloride of mercury, as has been previously reported.[9] Alkaline phosphatase activity in control animals was only slightly reduced after ligation for 48 hours and almost completely absent after one week.

COMMENT

Spermine is a potent nephrotoxin when administered parenterally to mice, rats, and rabbits. The lesion produced is a severe desquamative necrosis of the epithelial cells of the proximal convoluted tubules, which is capable of causing albuminuria, uremia, and death in a large number of animals, as has been previously reported by us.[10]

Microscopically, the spermine-induced lesion is not too dissimilar to that produced by other nephrotoxic agents, such as bichloride of mercury, uranium nitrate, potassium dichromate, and potassium chlorate. However, in mercurial nephrosis the principal site of the lesion is in the outer cortical or peripheral portion of the proximal convoluted tubule, whereas following spermine administration the distal portion of the proximal convoluted tubule is initially and most severely damaged although proximal portions are not spared later in the progress of the lesion. In addition, vascular changes have been noted in mercurial nephrosis.[11] No vascular changes have been recognized in spermine-induced renal disease. Uranium nitrate intoxication produces a lesion of the convoluted tubule similar to that produced by spermine, but progression into a chronic state occurs.[12] This has not been observed with spermine and shall be commented upon subsequently.

Ureteral ligation was performed to elucidate the pathogenesis of spermine-induced renal disease. Since it has been previously demonstrated that such a procedure suppresses glomerular filtration,[13] this phase of the study was undertaken to determine whether spermine acts at a critical plasma level or by means of tubular reabsorption or by direct action on the luminal surface of the epithelial cells of the proximal convoluted tubules. Little protection from the upper nephron nephrosis of spermine was afforded by the procedure, indicating that the nephrotoxic action of this agent is dependent upon a critical plasma level rather than on glomerular filtration. This is another difference noted between spermine-induced nephrosis and that resulting from bichloride of mercury administration, since no renal lesion is observed in the hydronephrotic kidney following administration of this latter agent.

We have been unable to produce a renal lesion with spermine that morphologically simulates spontaneous renal disease. Since spermine is a naturally occuring substance in mammalian tissues, the significance of such a relationship is obvious. Further, this agent is apparently unable to produce definite chronic lesions characterized by fibrosis or marked distortion of renal architecture, indicating that a tolerance is developed for spermine upon repeated injection.

The related amines, such as putrescine, cadaverine, and spermidine, have been found to be incapable of producing lesions in the animals studied. However, the production of renal lesions identical to those of spermine by the administration of the short-chain amines suggests that spermine action may be mediated through the products of its metabolism. It is interesting to note that medullary lesions have not been observed following the administration of any of the amines tested. However, lesions simulating human medullary necrosis following the administration of vinylamine have been reported by Mandel and Popper.[14] Earlier reports of this action

are also evident in the literature.§ Perhaps the difference in selective action of vinylamine from spermine and the other amines reported is due to the cyclical structure of the former.

SUMMARY

Spermine, an aliphatic amine that is found in many mammalian tissues, is capable of producing an upper nephron nephrosis resembling that produced by bichloride of mercury and uranium nitrate. However, the development of the lesion is not dependent upon glomerular filtration, as is the case with bichloride of mercury, but is dependent upon a critical plasma level. Other amines have been studied which produce renal lesions similar to that of spermine, suggesting that spermine action may be mediated through products of its metabolism. The lesion produced by spermine is different from that observed following the administration of the more extensively studied vinylamine.

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NEPHROTOXIC GLOBULIN NEPHRITIS

IV. Effect of Certain Biological Variables

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PREVIOUS papers in this series have discussed in detail the significance of variability in the manifestations of experimental nephritis, produced in the rat by administration of rabbit anti-rat-kidney gamma globulin (NTG). It was suggested that an analysis of variability might throw light upon the pathogenesis of this experimental disease, and a systematic attempt to isolate the possible causes of variability was undertaken. Previous reports have delineated the progress with time after a single intravenous NTG injection,[1] the relation of severity of the disease to adrenal size,* and the influence of antibodies to nonspecific components of the whole kidney antigen used for NTG production.[4]

Smadel and Farr,† Heymann and Lund,‡ Ehrich, Forman, and Seifer,[10] and others who have studied nephrotoxic serum nephritis used inconstant schedules of immunization for nephrotoxic serum production, inconstant schedules and routes of administration, mixed sexes, and animals of mixed ages (prepubescent and adult). Crude nephrotoxic serum was used in most of the reported work and, in some instances, for which the details of preparation are given, it is evident that the sera were not pooled before use. Since the variable production of antibodies in the immunized rabbit is well known, it seems probable that the nephrotoxic sera used were of inconstant potency and consequently could not be expected to give comparable results.

In the work reported here, we have analyzed the effects produced by certain variables upon the manifestations of nephrotoxic globulin nephritis. The variables considered are dose of NTG, potency of the NTG preparation, route of administration, and age and sex of the animals.

Ruth T. Lackey and Jay Banovitz gave technical assistance.

Dr. Campbell, Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena; Contribution No. 1854.

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* References 2 and 3.

† References 5-7.

‡ References 8 and 9.

METHODS AND MATERIALS

Nephrotoxic Globulin (NTG).—Purified nephrotoxic gamma globulin was prepared from rabbit anti-rat-kidney serum in the manner previously described.[1] Two lots were used, prepared from different lots of pooled rabbit serum. One lot (4/12/51) had a total protein concentration of 25.5 mg. per milliliter and was prepared from serum pooled from seven rabbits (No. 502 and 504 to 509) which had received kidney antigen injections twice a week to a total of 38 injections during the period from Nov. 30, 1950, to April 12, 1951. The second lot (8/9/51) had a total protein concentration of 23.0 mg. per milliliter and was prepared from serum pooled from 15 rabbits (No. 585 to 599) which had received kidney antigen injections twice a week to a total of 32 injections during the period from April 19, 1951, to Aug. 9, 1951.

Experimental Procedure.—145 rats of the Slonaker-Addis strain were used for these experiments. Adult males and females were selected to weigh 150 gm. each, with little variation, at the start of an experiment. Prepubescent males and females were selected to weigh 50 gm. each at the start of an experiment. Groups of 10 to 15 animals were used for each set of experimental conditions.

The animals which died as a result of the initial shock after intravenous NTG administration were discarded from the study, with the assumption that these immediate deaths were the consequence of antibodies to antigens not specific to the kidney.[4] At low dosage levels such deaths were infrequent, but at the highest dosage levels as many as 25% of the animals injected died within four hours.

Before the start of each experiment a blood pressure determination was made by the tail-microphone method of Friedman and Freed,[11] and a four-hour urine collection was made to determine the basal rate of protein excretion. Before and during the experiments the animals were fed the colony stock diet, which contained 17% protein, and received water ad libitum. During the periods of urine collection they received no solid food, but only 10% dextrose solution with 0.4% sodium chloride and 0.5% vitamin B complex elixir (Betaplexin).

During the four-week period of each experiment, blood pressure determinations followed by four-hour urine collections for protein analysis were made at weekly intervals. At the end of the fourth week an eight-hour urine collection was made. The animals were then killed by exsanguination from the severed abdominal aorta and vena cava during light ether anesthesia.

The following data were obtained: wet weight of kidneys, heart, adrenal glands, gonads, and liver. The organ weights were compared with the value predicted for a normal rat of the same size.§ The presence of ascites was noted, and, if present, the quantity of fluid was measured. The total drawn blood volume was measured, and the serum was examined for visible lipemia, which, when present, was graded from 1+ to 4+. The tissues were fixed, stained, and sectioned for microscopic examination as in our previous reports.

Protein content was determined in the individual urine specimens by the method of Shevky and Stafford.[14] Pools were made from aliquot portions of the urine and serum specimens in each group. Serum and urine urea determinations were performed by the method of Kibrick and Skupp.[15] Serum and urine total creatinine chromogen determinations were performed by the method of Bonsnes and Taussky.[16] Serum and urine chloride determinations were performed by the method of Schales and Schales.‖ Serum and urine total protein determinations were performed by the biuret method of Kingsley.[19] Serum total cholesterol determinations were performed by a slight modification of the method of Bloor, Pelkan, and Allen.[20] Serum total lipid determinations were performed by the method of Bloor.[21]

RESULTS AND COMMENT

General Observations.—The general behavior and appearance of rats that have been given nephrotoxic globulin (NTG) have been described previously in detail.¶ The same observations and comments pertain to this group of experiments, and the changes in behavior were grossly correlated with the dose and potency of NTG

§ References 12 and 13.

‖ References 17 and 18.

¶ References 1, 2, and 3.

administered. Because the data accumulated constitute an unwieldy mass, presentation and discussion here has been limited to those data which contributed new information, not previously reported in the earlier papers of this series. Microscopic examination of the tissues, serum and urine creatinine values, urine urea values, and serum and urine chloride values conformed with previous results and with the severity of the nephritis as graded by previously established criteria.#

Dose of NTG and Relative Potency of Preparations.—The two NTG preparations (4/12/51 and 8/9/51) were administered in graded amounts. As indicated above, the two preparations were identical, insofar as possible, except for a minor difference in the number of immunization injections and their derivation from different rabbit groups.

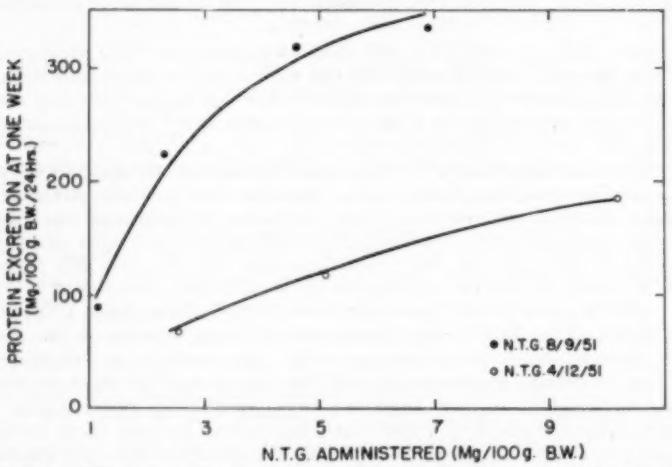

Chart 1.—Effect on protein excretion of variation in potency of NTG preparation and dose of NTG administered intravenously.

In Chart 1 the rate of protein excretion one week after a single intravenous NTG injection is plotted against the dose of NTG administered. In this experiment only adult male rats were used. The rate of protein excretion increased with the amount of NTG administered, but the increase was nonlinear. The divergence from linearity in this relationship was more pronounced with NTG (8/9/51), and at all levels of dosage NTG (8/9/51) induced a far greater rate of protein excretion than NTG (4/12/51).

As shown in Table 1, the liver, kidney, and adrenal gland weights increased with the proteinuria, although not in perfect concordance, and the relative organ weight increases at equivalent dose levels of NTG were greater with NTG (8/9/51). The serum urea and total cholesterol concentrations were elevated in all groups, but they were not significantly associated with the rate of protein excretion. In contrast, the serum total lipid concentration rose out of proportion to the rise in serum total cholesterol, in rough association with the rate of protein excretion, so that the total cholesterol/total lipid ratio fell from 0.21 to 0.12 as the amount of NTG (8/9/51) administered was increased. In all the groups the blood pressure

References 2 and 3.

determinations were slightly elevated compared with groups given control globulin, previously reported,* but there were no significant differences between the groups.

Prepubescent and Adult Rats.—In comparing prepubescent with adult rats, only NTG (8/9/51) was used, at a single dose level, 2.30 mg. per 100 gm. body weight. Comparison was made in separate groups for both sexes.

TABLE 1.—*Effect on Nephritis of Dose and Potency of Nephrotoxic Globulin (NTG)*

Rats, No.	Dose NTG, Mg./ 100 Gm.	Protein Exer. at 1 Wk., Mg./ 100 Gm./ 24 Hr.	% Increase Organ Wt.			Serum Concentrations				Final B. P.
			Liver	Adrenal	Kidney	Urea, Mg./ 100 Cc.	Cholesterol, Mg./ 100 Cc.	Lipid, Mg./ 100 Cc.	Protein,* %	
					NTG (8/9/51)					
10	1.15	89	11	10	7	26	52	252	5.16	138
12	2.30	223	34	20	50	64	134	987	5.18	143
12	4.60	318	57	57	88	60	108	845	3.68	130
10	6.90	335	51	36	121	40	126	1,172	4.47	154
					NTG (4/12/51)					
12	2.55	67	24	23	35	26	74	406	4.38	...
14	5.10	118	11	7	0	30	61	584	5.40	...
13	10.20	185	56	40	59	46	148	1,288	4.11	134

* In comparing total serum protein concentrations, it is necessary to allow for the fact that total serum protein concentration in the Slonaker-Addis rat increases with age and is always higher in the male than in the female of the same size.[22]

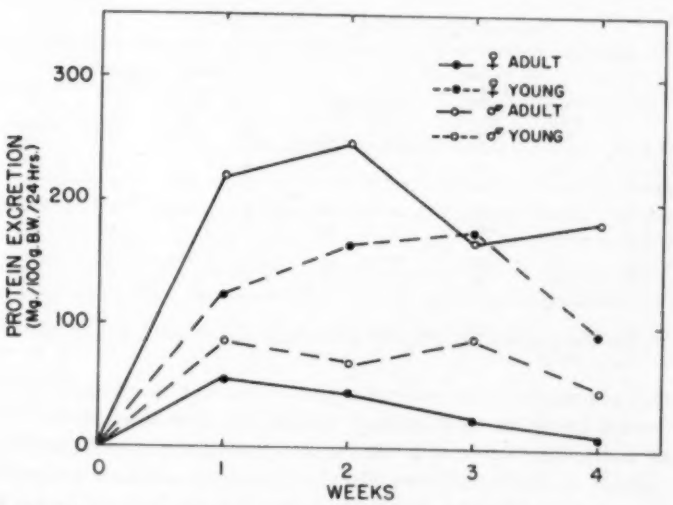

Chart 2.—Effect of sex and age (puberty) upon protein excretion after NTG administration.

The adult males showed a much greater degree of proteinuria than the prepubescent males throughout the experimental period (Chart 2). The organ weights were proportionately greater in the adult males (Table 2), along with the serum

* References 2 and 3.

urea, total cholesterol, and total lipid concentrations. The blood pressure in both groups was somewhat above the control values previously mentioned but was without a significant difference between the groups.

In females the situation was reversed. The prepubescent females had the more pronounced proteinuria and increase in relative adrenal gland and kidney weight. Liver weight could not be compared, because adequate standards are lacking for liver weight in the female rat of this strain. The prepubescent females also had a greater increase than the adult females in serum urea and total cholesterol concentrations. In both groups the blood pressure was little elevated, probably without significant difference from control values.

Sex Differences.—Adult male groups were compared with female groups at two different dosages of NTG (8/9/51) and at one dosage of NTG (4/12/51)

TABLE 2.—*Effect of Age on NTG Nephritis in the Rat*

Rats, No.	Sex	Age	Protein Exer. at 1 Wk., Mg./ 100 Gm./ 24 Hr.	% Increase Organ Wt.			Serum Concentrations				Final B. P.
				Liver	Adrenal	Kidney	Urea, Mg./ 100 Cc.	Cholesterol, Mg./ 100 Cc.	Lipid, Mg./ 100 Cc.	Protein, %	
12	M	Prepub.	87	28	— 2	11	34	73	270	4.73	151
12	M	Adult	223	34	20	50	64	134	987	5.18	143
14	F	Prepub.	122	..	16	44	60	93	...	4.82	130
12	F	Adult	54	..	—11	1	28	72	369	5.70	127

TABLE 3.—*Effect of Sex on NTG Nephritis*

Rats, No.	Sex	NTG Prep.	Dose NTG, Mg./ 100 Gm.	Protein Exer. at 1 Wk., Mg./ 100 Gm./ 24 Hr.	% Increase Organ Wt.		Serum Concentrations				Final B. P.
					Adrenal	Kidney	Urea, Mg./ 100 Cc.	Cholesterol, Mg./ 100 Cc.	Lipid, Mg./ 100 Cc.	Protein, %	
14	M	4/12/51	5.10	118	7	0	30	61	584	5.40	...
14	F	4/12/51	5.10	45	— 2	0	26	66	439	5.73	...
10	M	8/ 9/51	4.60	318	57	88	60	108	845	3.68	130
10	F	8/ 9/51	4.60	282	— 2	61	34	104	925	4.45	124
12	M	8/ 9/51	2.30	223	20	50	64	134	987	5.18	143
12	F	8/ 9/51	2.30	54	—11	1	28	72	369	5.70	127

in addition to the comparison of prepubescent male and female groups described above.

In the adult groups, the males showed a greater degree of proteinuria than the comparable female group in each instance (Table 3). The increase in relative adrenal gland and kidney size and in serum urea concentration and the drop in serum total protein concentration were likewise greater in the male groups than in the female groups. Differences in the serum total cholesterol and serum total lipid concentration were neither striking nor consistent. In the comparisons the male groups had slightly higher blood pressure, but in one pair of the groups compared the difference was not significant.

On the contrary, in the prepubescent groups the females had a higher rate of protein excretion, with consistent changes in the other recorded data, with one exception: here, also, the males had higher blood pressure.

Route of Administration.—Comparisons were made in male rats, using NTG (4/12/51) at a single dose level, 5.10 mg. per 100 gm. body weight. One group (IV) received a single intravenous NTG injection at the start of the experiment. A second group (SC) received a daily subcutaneous NTG injection for the entire four-week period of the experiment. A third group (SC-IV) received a daily subcutaneous NTG injection for the entire period, but on the 21st day, only, the dose was given intravenously instead of subcutaneously.

As shown in Table 4, Group IV had a pronounced proteinuria at the end of one week, while the other two groups had no increase from the normal slight proteinuria of the rat. At the end of four weeks, Group IV had twice the proteinuria of Group SC. However, Group SC-IV had the greatest proteinuria of the three groups, twice as great as the proteinuria of Group IV at one week, which is a more reasonable basis for comparison.

Because of the different time intervals after intravenous injection, the relative organ weights of Group SC-IV are not comparable to those of the other two groups,[1] but it is interesting to note that, at four weeks, the relative liver and kidney weights of Groups IV and SC were similar but the relative adrenal weight in Group SC was much greater than the relative adrenal weight in Group IV.

TABLE 4.—*Effect of Route of Administration on NTG Nephritis*

Rats, No.	Group	Protein Excretion, Mg./100 Gm./24 Hr.			% Increase Organ Weight		
		At 1 Wk.	At 3 Wk.	At 4 Wk.	Liver	Adrenal	Kidney
14	IV	118	70	33	11	7	0
12	SC	2	9	17	7	29	1
12	SC-IV	2	23	225	30	36	33

COMMENT AND CONCLUSIONS

The result of this study is to show that certain biological variables, dosage, potency of the NTG preparation, route of administration, and age and sex of the animals, all influence greatly the course and manifestations of nephrotoxic globulin nephritis. Since these variables have not all been controlled in many of the previous reports in this field, the results and conclusions in some publications are rendered doubtful. Final opinions should await reexamination of the original data or repetition of the work under satisfactory conditions, with more carefully controlled conditions.

It is not surprising to find the differences in potency between preparations described above. Determination of the minimal precipitable antibody in nine different lots of NTG gave results which varied from 6.5 to 16.0%. While it is certainly true that the minimal precipitable antibody content of an antikidney globulin preparation, measured with only the saline-soluble kidney material as a precipitating antigen, does not measure the entire antikidney potency present, the wide variation in the values obtained certainly renders reasonable the belief that the preparations are dissimilar. Consequently the total antikidney potency may vary widely in a similar fashion, a belief which is further supported by the data given in this paper.

The route of administration requires serious consideration. The differences produced by the route of administration, while not surprising, were of striking magnitude. The SC group, which showed changes of the same order of magnitude as, but less than, those in the IV group at four weeks, received 28 times the dose of NTG received by the IV group. The difference in result might be due to incomplete absorption, with destruction of the NTG at the subcutaneous site of administration before it could reach the kidney, due to the alteration of NTG either by metabolic or immunologic means, or due to an exposure of the kidney to continuous infusion of NTG in low concentration from the subcutaneous site, compared with the sudden flood of NTG received by the kidney from an intravenous injection. At three weeks, Group SC-IV must have had renal damage which rendered the intravenous dose more damaging than when administered without the subcutaneous "priming." Nevertheless, after three weeks of subcutaneous NTG injections both Groups SC and SC-IV had little increase from the normal basal rat proteinuria. The increased relative adrenal size in Group SC, compared with Group IV, may be the result of daily handling and pain from the subcutaneous injection, since Group IV was not given a daily injection of normal control rabbit globulin, which would have been necessary to render the conditions completely comparable.

A pronounced sex difference in response to NTG has been shown in the adult. This sex difference is reversed before puberty, with the exception of a possible difference in blood pressure elevation, the males having a slightly higher blood pressure after NTG administration, of doubtful significance, in both adult and prepubescent groups. It might be expected that the sex difference would develop with puberty, but it was surprising to find a reversal of the difference in the prepubescent rat. The adult female Slonaker-Addis rat has a much larger adrenal gland than the male, but this sex difference does not develop until puberty, at a body weight of approximately 100 gm.† It is interesting to note that, while in each sex considered separately the relative adrenal weight is directly correlated with the severity of nephritis,‡ the female adult, with an adrenal gland which is naturally larger than that of the male, has less severe nephritis under comparable conditions.

The dosages used were calculated on the basis of body weight, a matter of no concern except in the comparison of adult and prepubescent rats, since all the adults were chosen to weigh 150 gm. It may be suggested that the dosage might better have been calculated on the basis of estimated kidney weight. However, this substitutes an estimate for a direct measurement, and the differences observed in the adult and prepubescent rats are sufficiently great to render the basis for calculation of minor importance.

SUMMARY

The effects produced by certain variables upon the manifestations of nephrotoxic globulin nephritis in the rat have been studied. It was found that preparations of nephrotoxic globulin (NTG) made under similar conditions have widely variable antikidney potency. The severity of the nephritis increases in nonlinear relation to the dose of NTG, and the degree of nonlinearity may vary between preparations. The route of administration influences greatly the effect of a given dose of NTG.

† References 12 and 13.

‡ References 2 and 3.

There is a pronounced sex difference in the severity of nephritis produced when the other factors are held constant. The adult male responds with a much more severe nephritis than the adult female. Puberty influences the sex difference greatly, and, in contrast to the adult sex difference, the prepubescent female responds with a much more severe nephritis than the prepubescent male. The effect of these biological variables must be controlled in order to study the effect of other factors on the pathogenesis of nephrotoxic globulin nephritis in the rat.

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News and Comment

Professional Education Color Television Broadcasts.—The American Cancer Society, in conjunction with the facilities of the Columbia Broadcasting System Television Network, will produce during 1954 a series of closed-circuit color television programs for physicians. The following are the locations of the Giant Screen Receivers for these Telecolor Clinics:

New York, Hosack Hall, New York Academy of Medicine, 2 E. 103d St.
Boston, Auditorium, Children's Cancer Research Foundation, 35 Binney St.
Toledo, Ohio, Auditorium, Academy of Medicine Building, 3101 Collingwood Blvd.
Philadelphia, WCAU Auditorium, City & Monument Aves.
Pittsburgh, Mellon Institute, 4400 Fifth Ave.
Detroit, Auditorium, Masonic Temple, 500 Temple Ave.
Dearborn, Mich., Ford Rotunda, Schaefer Highway & Rotunda Dr.

The programs for 1954 cover the following topics:

Date	Title	Representative
1/6/54	The Diagnosis and Treatment of Uterine Cancer	Dr. Howard C. Taylor Jr.
1/13/54	Sarcoma of Soft Parts	Dr. Arthur Purdy Stout
1/20/54	Cancer of the Oral Cavity	Dr. Maurice Lenz Dr. Ernest Daland
1/27/54	Cancer of the Larynx and Hypopharynx	Dr. Maurice Lenz
	Cancer of the Thyroid	Dr. William B. Parsons
2/3/54	Cancer of the Skin	Dr. Arthur Purdy Stout Dr. Thomas Stevenson Dr. Maurice Lenz
	Cancer of the Central Nervous System	Dr. Lawrence Pool Dr. Thomas Bridges
2/10/54	Lymphomas and Leukemias	Dr. Alfred Gellhorn Dr. Ruth Guttman
2/17/54	The Management of Advanced Cancer	Dr. Alfred Gellhorn Dr. Maurice Lenz Dr. Thomas Bridges
2/24/54	Cancer Detection	Dr. Emerson Day
3/3/54	Head and Neck Cancer	Dr. Hayes E. Martin
3/10/54	Hormone Therapy in Inoperable and Recurrent Breast Cancer	Dr. Norman E. Treves
3/17/54	Cancer of the Lung	Dr. William L. Watson
3/24/54	Significance of Indigestion	Dr. George T. Pack
3/31/54	Advances in Control of Cancer of Colon and Rectum	Dr. George E. Binkley
4/7/54	Treatment of Recurrent Cancer of the Cervix	Dr. Alexander Brunschwig
4/14/54	Cancer of the Genito-Urinary Tract	Dr. Willet E. Whitmore
4/21/54	Bone Tumors	Dr. Bradley E. Coley
4/28/54	Melanoma	Dr. George T. Pack
5/5/54	Lymphoma and Leukemia	Dr. Lloyd F. Craver
5/12/54	Tumors of Childhood	Dr. Harold W. Dargeon
5/19/54	Hormonal and Chemical Treatment of Cancer	Dr. Rulon W. Rawson
5/26/54	Treatment of Patients with Advanced Cancer	Dr. Raymond Houde
	Psychiatry and Analgesia	Dr. Arthur M. Sutherland
6/2/54	Frontiers of Research	Dr. C. P. Rhoads

American Goiter Association.—The 1954 meeting of the American Goiter Association will be held at the Somerset Hotel, Boston, April 29 and 30, and May 1, 1954.

The program for the three-day meeting will consist of papers and discussions dealing with the physiology and diseases of the thyroid gland.

Conference on Coroners' Problems.—A Conference on Coroners' Problems will be presented by the University of Minnesota from March 8 to 10, 1954, at the Center for Continuation Study on the University Campus. Intended primarily for coroners, the course will also be of interest to law enforcement officers and to pathologists. Procedures of help in the investigation of all types of accidental or suspicious deaths will be discussed. The faculty for the course will include Dr. Walter W. Jetter, Professor of Legal Medicine, Boston University, and the program will be presented under the direction of Dr. James McCartney, Professor, Department of Pathology, University of Minnesota Medical School. Lodging and meal accommodations are available at the Center for Continuation Study.

Appointments.—Benjamin Castleman, M.D., has been appointed Clinical Professor of Pathology at Harvard Medical School and Chief of the Department of Pathology at the Massachusetts General Hospital. Dr. Castleman has been associated with the latter institution since 1931 and with Harvard Medical School since 1935.

Philipp R. Rezek, M.D., has been appointed Professor of Pathology in the University of Miami School of Medicine. He continues as Director of Pathologic Anatomy at the Jackson Memorial Hospital in Miami.

ANNOUNCEMENTS

Meetings.—The 1954 Annual Meeting of the American Society for Experimental Pathology will be held with other constituent societies of the Federation of American Societies for Experimental Biology in Atlantic City, N. J., April 11-16, 1954.

The Second National Conference on Trichinosis will be held in the Auditorium of the American Medical Association, 535 N. Dearborn St., Chicago, on Monday, March 1, 1954. The purpose of the conference is to discuss methods of education, problems of human and animal health, and research in relation to control of this disease. S. E. Gould, M.D., of Wayne County General Hospital, Eloise, Mich., serves as Chairman, Continuing Committee on Trichinosis.

Richard H. Jaffe Memorial Lecture.—The Fifth Richard H. Jaffe Memorial Lecture of the Institute of Medicine of Chicago was given by Paul E. Steiner, M.D., Professor of Pathology, University of Chicago. Dr. Steiner's topic was "Etiological Factors in Cancers as Revealed by Ethnic and Geographical Studies."

Fellowship Awarded to Dr. Fallis.—The 1954 Sarah Mellon Scaife Fellowship in Pathology at the University of Pittsburgh has been awarded to Bruce D. Fallis, who is at present instructor in physiology and biochemistry at the University of Texas School of Medicine, Galveston, Texas.

Books

Hepatitis. Transactions of the International Society of Geographical Pathology, Liège, July 15-18, 1953. Published separately, from the Schweizerische Zeitschrift für allgemeine Pathologie und Bakteriologie (**16**:259-652, 1953). Price, 46.80 Swiss francs. Pp. 393, with several illustrations and tables. S. Karger, Holbeinstrasse *22*, Basel, 1953.

Excerpta Medica, Section XVI: Cancer (Experimental and Clinical). Vol. 1, (July-Dec., 1953). Price, $5.00. Vol. II, (1954). Price, $10. Excerpta Medica, 111 Kolverstraat, Amsterdam, 1953.

An Atlas of Pelvic Operations. By Langdon Parsons, M.D., Professor of Gynecology, Boston University School of Medicine; Chief, Department of Gynecology, Massachusetts Memorial Hospital; Gynecologist, Palmer Memorial Hospital; Gynecologist, Massachusetts Department of Public Health, Hospital for Cancer at Pondville, Mass., and Howard Ulfelder, M.D., Clinical Associate in Gynecology, Harvard Medical School; Assistant Surgeon, Massachusetts General Hospital; Gynecologist, Massachusetts Department of Public Health, Hospital for Cancer. Illustrations by Mildred B. Codding, A.B., M.A. Price, $18. Pp. 231, with 197 plates. W. B. Saunders Company, 218 W. Washington Sq., Philadelphia; W. B. Saunders, Ltd., 7 Grape St., Shaftesbury Ave., London, 1953.

Annals of the New York Academy of Sciences: Volume 56, Article 6, The Relation of Lean Body Weight to Metabolism and Some Consequent Systematizations. By Captain Albert R. Behnke, (MC), U.S.N., Medical Research Laboratory, United States Submarine Base, New London, Conn. Price, $1.25. Pp. 48, with 11 figures. New York Academy of Sciences, American Museum of Natural History, Central Park W. and 77th St., New York, 1953.

Annals of the New York Academy of Sciences: Volume 56, Article 5, Growth of Protozoa. By S. H. Hutner and 52 other authors. Price, $4.50. Pp. 280, with several figures and illustrations. New York Academy of Sciences, American Museum of Natural History, Central Park W. and 77th St., New York, 1953.

Die Kreuzschmerzen der Frau: Ihre Deutung und Behandlung; Gynäkologische Orthopädie. By Heinrich Martius, M.D., Director of the Women's Clinic of the University, Göttingen, Germany. Fourth Revised Edition. Price, DM 19.50. Pp. 166, with 73 illustrations. Georg Thieme, Diemershaldenstrasse 47, (14a) Stuttgart, Germany; agents for U. S. A.: Grune & Stratton, Inc., 381 Fourth Ave., New York 16, 1953.

Handbuch der inneren Medizin: Volume III. Edited by G. von Bergmann, M.D., W. Frey, M.D., and H. Schwiegk, M.D. Verdauungsorgane (Part 1). By W. Baumann, K. Beckmann, A. Gigon, M. Gülzow, N. Henning, G. Katsch, M. Lüdin, and H. Pickert. Price, DM 130. Pp. 941, with 283 illustrations, some of which are in color. Springer-Verlag, Berlin, 1953.

A comprehensive work covering all branches of gastroenterology, printed on excellent paper and well illustrated. Roentgenograms are of remarkable technical quality. Numerous colored diagrams, in particular of gastroscopic and peritoneoscopic findings are well done. Each major chapter has been written by different authors, most of them well known in the field of gastro-

enterology. According to the old tradition of this encyclopedic work, the older literature is extensively quoted in the text and the majority of papers discussed had been published prior to World War II. The American literature is fairly well represented, and most of the sections are up-to-date. Still too much emphasis is placed on work done in the late 19th century and the early 20th century, which today is of historic interest only. In the reviewer's opinion the discussions would be less involved and easier to digest if some of the older papers had been eliminated or placed in the section on the history of the particular subject. Undue emphasis is placed on certain aspects in the field. For example, gastroptosis is discussed on six pages as a pathological entity. Sprue, on the other hand, covers also approximately six pages only, and the discussion of this subject appears inadequate. Electrolyte changes in severe diarrheic conditions are mentioned only casually, and measures to maintain adequate electrolyte balance are nearly disregarded. The potassium problem in gastrointestinal conditions is hardly touched upon. The role of bacteria (e. g., staphylococci in acute gastroenteritis) is minimized. Two pages only are devoted to segmental ileitis. The section on liver diseases is extensive and adequately covered, although well-established facts are as broadly described as unsubstantiated assumptions. Baumgaertel's work, which has contributed new aspects to our knowledge of bilirubin metabolism, is well discussed. On the whole, the book can be recommended to those readers who are experienced enough to recognize the important features and discard unsubstantiated claims. The American student of the subject will find little which would add to the knowledge acquired in this country during the past two decades.

Standard Methods of Clinical Chemistry: Volume 1. By the American Association of Clinical Chemists. Editor-in-Chief, Miriam Reiner, Director, Chemistry Laboratory, Gallinger Municipal Hospital, Washington, D. C. Price, $4.50. Pp. 142. Academic Press Inc., 125 E. 23rd St., New York, 1953.

This 142-page volume presents in detail a method or methods of performing 17 different examinations. Included is a review of the flame photometer. Each method published has been submitted by a responsible laboratory. The method has then been tested by a responsible "checking" laboratory. Methods have been chosen on the basis of accuracy and simplicity. The publication may serve to place readily into the hands of the clinical pathologist a satisfactory method of performing the test desired. Because of the wide variance of problems confronted by various laboratories, the methods submitted may not be the method of choice in all instances. Of practical value is the inclusion of discussions and precautions accompanying each method.

Peripheral Nerve Injuries: Principles of Diagnosis. By Webb Haymaker, M.D., Chief of Neuropathology Section, Armed Forces Institute of Pathology, Washington, D. C., and Barnes Woodhall, M.D., Professor of Neurosurgery, Duke University School of Medicine, Durham, N. C. Second Edition. Price, $7.00. Pp. 333, with 272 illustrations. W. B. Saunders Company, 218 W. Washington Sq., Philadelphia 5; W. B. Saunders Company, Ltd., 7 Grape St., Shaftesbury Ave., London, W.C. 2, 1953.

This edition is intended for students and physicians who might not be previously trained in the recognition of peripheral nerve injuries. The opening chapters deal with the minute and gross anatomy of motor, sensory, and sympathetic nerve fibers in the peripheral nerves. Sensory dermatome charts are presented with preference for those of Foerster, which are considered to be most useful clinically. There are detailed charts of the innervation of the various muscles, skin, and parts of the skeleton. The second large section of the book deals with examination of the peripheral nervous system with details of the anatomy of movement so important in the accurate diagnosis of peripheral nerve injuries. "Trick" movements are also described. Chapter 7 deals with different degrees of nerve injury in the terms of changes which may be seen by pathological study. Microphotographs of representative changes illustrate alterations which range from simple demyelinization to fifth degree injury, in which there is total loss of motor, sensory, and sympathetic function with discontinuity of the ends of the severed nerve. A fuller discussion of neuropathology of peripheral nerve injuries is not given because it is available in the "Atlas of Peripheral Nerve Injuries" by Lyons and Woodhall. The following chapter describes the changes in muscles, sensation, reflexes, sympathetic innervation, and blood vessels due to or

accompanying nerve injuries. Various types of contractures are illustrated. Subsequent sections deal with special tests for nerve function and regeneration (such as Tinel's sign and electrical stimulation tests) and the remainder of the book takes up in detail the specific injuries of peripheral nerves and their plexuses. The subtitle "Principles of Diagnosis" indicates the authors' intention in writing the book which, therefore, omits details of treatment and the results thereof. This book should be of great value to those who treat peripheral nerve injuries and to diagnosticians in general.

Annals of the New York Academy of Sciences: Volume 57, Article 3, Ion Exchange Resins in Medicine and Biological Research. By Harry Sobotka and 26 other authors. Price, $4.50. Pp. 262, with several figures and numerous tables and charts. New York Academy of Sciences, American Museum of Natural History, Central Park W. and 77th St., New York, 1953.

Bakteriologische Nährböden: Ausgewählte Nährbodenrezepturen für das medizinisch-bakteriologische Laboratorium. By Dr. Lothar Hallmann, Hamburg, Germany. Price, DM 19.80. Pp. 252, with 52 illustrations. Georg Thieme, Diemershaldenstrasse 47, Stuttgart, Germany; agents for U. S. A.: Grune & Stratton, Inc., 381 Fourth Ave., New York 16, 1953.

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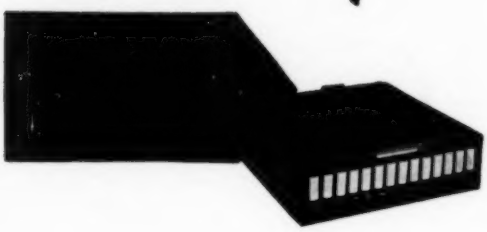

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